Plasmodium life-cycle specific protection and humoral responses against malaria after chemoprophylaxis and sporozoite immunization

Wiebke Nahrendorf

Plasmodium life-cycle specific protection and humoral responses against malaria after chemoprophylaxis and sporozoite immunization

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Contents

CHAPTER 1

Preface and introduction

Scope of this thesis

Preface

The focus of this thesis is protective antimalarial immunity induced by chemoprophylaxis and sporozoite immunization. To appreciate why we need to understand immunity elicited by this experimental malaria immunization, the following introductory chapter elucidates the background of global malaria burden; the *Plasmodium* parasites causing it and the problems faced by efforts to eradicate malaria. The second part discusses the acquisition of antimalarial immunity, in particular humoral immunity. Different rodent malaria species and their contribution to our understanding of malaria are introduced. The need for an effective malaria vaccine to combat this global health scourge is highlighted and an overview of currently pursued approaches is given. The *Plasmodium chabaudi* mouse model and controlled human malaria infections are described in detail. Both approaches are used to study the objectives of this thesis i.e. to unravel the stage-specificity of protection and the contribution of humoral immunity after immunization with sporozoites and chloroquine.

Introduction

The burden of malaria

Over 3 billion people, close to half of the world's population, are at risk of falling ill from malaria[1]. Over the course of the previous century, developed countries with temperate and sub-tropical climate in North America and Europe have eliminated malaria. Today mainly people living in poverty in tropical countries of Africa, Asia and South America suffer from the disease[2] (**Figure 1**). In addition to 207 million cases and a death toll of approximately 627,000 (World Health Organization (WHO) estimate for 2012[3]), most of which occur in children under 5 years of age in Sub-Saharan Africa, malaria also has enormous socio-economic consequences[4]. Poverty is concentrated in countries that are malaria-endemic and the extent of this correlation suggests they are intimately related. In Uganda malaria morbidity leads to an annual loss of 49.8 Million US$ of the countries gross domestic product thereby impairing long-term economic growth and efforts to eradicate poverty[5]. To combat this global scourge the stated 6th goal of the United Nations Millennium Declaration is therefore to eliminate malaria as a major cause of mortality and reduce its impact on socio-economic development by 2015[6]. Efforts made by the Roll Back Malaria partnership and other research and public health coalitions have lead to a 25% decline in global cases, and deaths from malaria have fallen by 42% since 1998[7]. A combination of interventions including timely diagnosis, treatment with effective drugs and vector-control by indoor spraying and bed net use have contributed to this decline in malaria incidence, but resistance of parasites and mosquitoes to drugs and insecticides is emerging. In addition, asymptomatic carriers are a constant source for ongoing transmission to the mosquito vector[8]. New strategies, including the development of an effective malaria vaccine, have hence to be pursued to continue reducing (and eventually eliminating) the global malaria burden.

The life-cycle of *Plasmodium*

The geographic distribution of malaria is restricted to areas where the mosquito vector *Anopheles* can thrive. Year-round high temperatures combined with rainfall and humidity provide ideal breeding-conditions for more than 400 *Anopheles* species, 25 of which are anthropophilic and thus effectively transmit malaria to humans[9]. The discovery of the apicomplexan malaria parasite *Plasmodium* in the salivary glands of tropical mosquitoes earned Ronald Ross a Nobel Prize

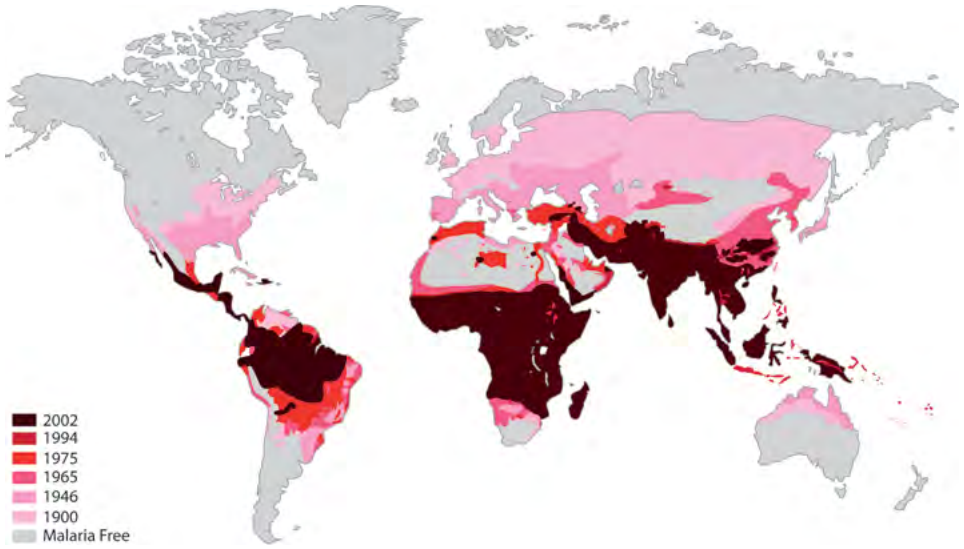

Figure 1 | Global malaria endemicity between 1900 and 2002
Global malaria distribution maps from pre-intervention (circa 1900) over the course of the 20th century until 2002. High and low risk areas are merged for each time point to illustrate all-cause malaria transmission distribution.
Reproduced with permission by the publisher from Hay *et al*., 2004[1]

in 1902[10]. Five years later Alphonse Laveran also received the Nobel Prize by confirming that the presence of the parasite in the blood causes the disease[11].
Wild *Anopheles* mosquitoes typically harbour between 850 and 4000 sporozoites in their salivary glands[12,13], but experimental models have shown that typically few (as little as 1 to 100) motile sporozoites are injected into the skin during one infectious bite[14,15] (**Figure 2**). It appears that a substantial number of sporozoites remains in the dermis for up to 6h and about ⅓ of the inoculum enters lymphatic vessels[16]. Sporozoites that reach the blood circulation migrate to the liver where they traverse through several sinusoidal and Kupffer-cells as well as hepatocytes before settling on a final cell[17-19]. Over the next 2-10 days (dependent on the *Plasmodium* species) the uni-nucleated sporozoite undergoes an enormous asexual replication called schizogony to form 10,000 to 30,000 merozoites, that get released into the bloodstream as merosomes surrounded by a temporal membrane[20,21]. Some species of malaria can form hypnozoites, dormant stages of liver-stage parasites that can get re-activated to undergo schizogony at any time. Once in the blood stream each merozoite invades one erythrocyte that, upon maturation, can release 6 to 20 (dependent on *Plasmodium* species) new

merozoites, which can in turn multiply leading to exponential growth in non-immune individuals[22,23].
By a poorly understood mechanism, some blood-stage parasites develop into gametocytes; the sexual form of the parasite, which can be taken up during a mosquito blood meal. Male and female gametes fuse and undergo meiosis forming a diploid zygote in the mosquito midgut. This zygote then differentiates into a motile ookinete that penetrates the gut-wall and resides on the exterior gut-wall forming an oocyst[24]. This process is the most substantial bottleneck in the life-cycle with typically as few as 2 oocysts formed per mosquito. Inside the oocyst thousands of haploid sporozoites are produced that can then migrate actively to the salivary glands of the mosquito, starting the life-cycle again.

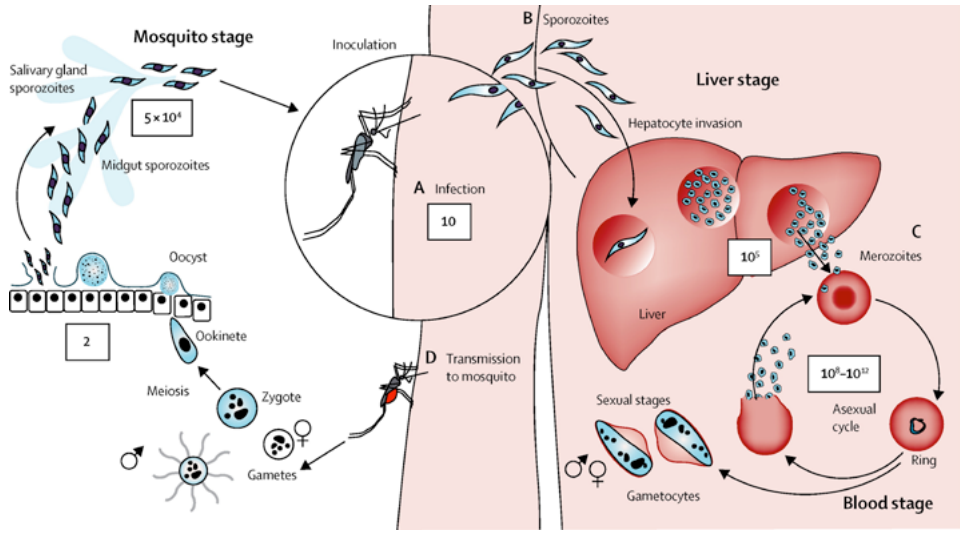

Figure 2 | The life-cycle of *Plasmodium*
A) During natural mosquito bite infection in endemic areas a female *Anopheles* mosquito inoculates approximately 10 sporozoites into the dermis, some of which reach the liver to establish an infection of hepatocytes. **B)** Inside heapotcytes the parasite undergoes an immense asexual replication called schizogony for 2-10 days (dependent on *Plasmodium* species), multiplying so one infected liver cell can give rise to between $1x10^4$ and $3x10^4$ merozoites. **C)** After approximately 10^5 merozoites get release from hepatocytes, they invade erythrocytes and mature (becoming first rings, then trophozoites, then schizonts) and asexually replicate so each blood-stage schizont can give rise to 6-20 (dependent on *Plasmodium* species) new merozoites. This exponential blood-stage replication causes all malaria pathology. **D)** By a poorly understood mechanism some blood-stage parasites develop into male or female gametocytes, the stage that is transmissible to the mosquito. After being picked up by a feeding mosquito the gametes fuse, forming diploid zygotes, which become motile ookinetes traversing the midgut wall of the mosquito and residing just outside the gut as round oocysts. Despite there typically being only very few oocyts (2) they can give rise to $5x10^4$ sporozoites after haploid replication. These sporozoites actively migrate to the salivary glands awaiting inoculation into a vertebrate host upon the next feed.
Reproduced with permission by the publisher from White *et al*., 2014[29]

The complexity of the malaria life-cycle makes it difficult to find universal targets for intervention, and recombination in the mosquito intensifies genetic drift of parasite populations in the field[25]. On the other hand, the decrease in parasite numbers to single digits in certain life-cycle stages (infected hepatocytes, ookinetes and oocysts) could be exploited for better parasite control (**Figure 2**).

***Plasmodium* species causing malaria in humans and disease manifestations**

The apicomplexan malaria parasite *Plasmodium* has co-evolved with the mosquito vector and its host for millions of years and can infect many vertebrates including birds, reptiles and mammals. Host specificity is very strict; for example *P. reichenowi*, which causes malaria in chimpanzees, fails to infect humans[26]. Five *Plasmodium* species with varying global distribution and infection characteristics cause malaria in humans; namely *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi* (**Table 1**). Infections with *P. falciparum* are of major concern, since this parasite is common all over the tropics, especially in Africa, causing severe disease and the greatest mortality of the five species. *P. vivax* was prevalent in temperate areas of Europe and North America until the second half of the 20th century and still occurs frequently in South America and Asia. It can cause febrile illness but leads to death less frequently than *P. falciparum*. *P. vivax* and *P. ovale*, which is very rare outside West-Africa, form hypnozoites (dormant stages of the parasite residing in the liver), which can lead to multiple relapse-infections for up to 1 year after the initial mosquito bite[27,28]. Infections with *P. malariae* occur all over the tropics, but are infrequent and usually mild, therefore having little impact on public health compared with *P. falciparum* and *P. vivax*[29]. *P. falciparum* can be cultured continuously in human blood under laboratory conditions since 1976, the importance of which for malaria research cannot be overestimated[30] (**Table 1**).

P. knowlesi was first described in 1931 in a long-tail macaque (*Maccaca fascicularis*) and can infect other monkeys in the wild and experimentally[31]. After circumstantial reports that *P. knowlesi* can cause malaria in humans[32], a startling study by Singh *et al.*[33] demonstrated that over half of all malaria cases in the Malaysian part of Borneo could be attributed to *P. knowlesi*, which had been microscopically misdiagnosed as *P. malariae*. Infections with this parasite are concentrated in Malaysia and adjacent countries and can lead to severe disease and death. Reports about emerging resistance to chloroquine and mefloquine are a further source of concern[34]. *P. knowlesi* can now be successfully cultured and genetically modified in the laboratory in human blood[35], which will greatly facilitate

Table 1 | Characteristics of the five *Plasmodium* species causing malaria in humans

	P. falciparum	*P. vivax*	*P. ovale*	*P. malariae*	*P. knowlesi*
Geographical distribution	Pan-tropical, most common in Africa	Pan-tropical, most common in Asia and South America	Africa, most common in West-Africa	Tropical regions world-wide	South-East Asia, most common in Malaysia
Global prevalence	High	High	Low	Low	Low (focally high)
Proportion where endemic	80-90%	50-80%	5-8%	0.5-3%	1-60%
Risk of mortality	High	High	Low	Low	High
Risk of drug-resistance	High	High	Low	Low	High
Relapse- formation of hypozoites	No	Yes	Yes	No	No?
Erythrocyte preference	Normocytes and reticulocytes	Reticolucytes	Normoctes	Reticolucytes	Normocytes and reticulocytes
Duration of one blood-stage cycle	48h	48h	48h	72h	24h
Continuous *in vitro* culture system	Yes	No	No	No	Yes
Animal reservoir	No	No	No	No?	Yes, monkeys

important research on this emerging human pathogen.

After mosquito bite the infection with malaria parasites remains asymptomatic during the liver-stage replication and the first erythrocytic cycles. *P. falciparum* and *P. knowlesi* non-selectively invade young and old erythrocytes and can thus reach high parasite densities. *P. ovale* only targets normocytes while *P. vivax* and *P. malariae* selectively invade reticulocytes[36] (**Table 1**). The length of one erythrocytic replication cycle varies from 24h for *P. knowlesi* to 48h for *P. falciparum*, *P. vivax* and *P. ovale* to 72h for *P. malariae* (**Table 1**). Blood-stage infections can persist for months or years (decades for *P. malariae*) when untreated, as new antigenic variants escape control by the immune system[37]. Symptoms are caused directly by the parasite and by inflammatory host immune responses. Patients suffering from uncomplicated malaria have flu-like symptoms including head- and muscle-ache as well as fever[29]. Life-threatening manifestations of malaria include severe anaemia, which results from rupture of parasitized red blood cells and decreased erythropoiesis[38] and acute respiratory distress syndrome[39]. *P. falciparum* parasitized erythrocytes are able to agglutinate[40], rosette[41] and adhere to endothelial cells to escape clearance by the spleen, causing severe complications by obstructing the microvasculature of vital organs, e.g. in the placenta[42]. Parasite accumulation in the brain combined with strong inflammatory responses can lead to the development of cerebral malaria, characterised by seizures, coma and death[43].

Malaria eradication efforts and the emergence of drug and insecticide resistance

Control and elimination of malaria has been achieved in some parts of the world mainly by controlling the vector i.e. by using bed nets, insecticide spraying, and environmental control measures such as larvaciding and filling and draining of breeding sites[44]. These interventions contributed substantially to malaria elimination from the United States between 1947 and 1951. Encouraged by this success and armed with the effective, long-lasting insecticide dichlorodiphenyldichloroethane (DDT) and the antimalarial drug chloroquine, the WHO launched a global malaria eradication campaign in 1955[45]. Eradication efforts initially focused on South-East Asia and South America with plans to include Sub-Saharan Africa later[45]. The campaign was driven by the Macdonald equation for vector control that predicts that reduction of mosquito population size below a critical threshold would disrupt transmission[46]. In addition, chloroquine was administered on a mass scale, and

even added to table salt in some countries[47]. Primaquine, the only drug that can eliminate dormant liver-stage parasites and mature *P. falciparum* gametocytes[48], had just been introduced and was used to treat *P. vivax* and *P. ovale* infections and reduce transmission further. After initial success for example in India and Sri Lanka, resistance to DDT and chloroquine as well as organisational problems led to the failure of the WHO campaign, which was abandoned in 1969. Consequences were catastrophic. Because clinical immunity is only acquired and maintained after multiple previous exposures (**Figure 3**), India and Sri Lanka suffered a devastating malaria epidemic with over 6 million cases in 1976[49]. This was facilitated by an abrupt increase in vector numbers as *Anopheles* mosquitoes became resistant to the insecticide DDT by a mutation in a sodium channel, which also mediates resistance to other insecticides, compromising vector control all over the world to this day[50]. Furthermore chloroquine-resistant *P. falciparum* (and to a smaller extend *P. vivax*) parasites emerged[51], which was accelerated by mass administration of chloroquine monotherapy at often subcurative dosages[52]. Chloroquine resistance was first reported in 1960 in Thailand[53] and Columbia[54] and reached Africa almost 20 years later in 1978[55], causing millions of deaths.

In the 1990s, resistance to most antimalarial drugs such as chloroquine and sulfadoxine-pyrimethamine had evolved and worsened the burden of malaria across the globe. With the imminent prospect of untreatable malaria infections in South-East Asia, the introduction Artemisinin, a compound that was identified in Chinese traditional medicine, saved control efforts by substantially lowering morbidity and mortality. Artemisinin-based combination therapies are the WHOs recommended therapy for all *P. falciparum* infections since 2005. Together with vector control, in particular widespread insecticide-treated bed net usage, this rapidly acting drug has contributed significantly to control malaria. Recently, however, resistance against Artemisinin has spread across South-East Asia[56], originating from the same area as chloroquine resistance in previous decades. It was suggested that the unusual genetic structure of parasites in this region[57] as well as Artemisinin monotherapy, poor drug quality, fake drugs as well as subcurative dosages have facilitated development of resistance, which is mediated by a single nucleotide polymorphism in *kelch 13*[56]. Spread of resistant *P. falciparum* parasites to Africa would reverse the gains made for malaria control in recent years, which makes some call for radical measures to confine Artemisinin resistance to Asia[58]. The combination of vector control with surveillance (using rapid diagnostic tests and PCR) and drug-treatment (especially Artemisinin-based combination therapies) have already lead to a substantial decline in malaria cases and mortality in recent

years[3]. It is however questionable that these tools will be sufficient to eradicate malaria. Since it is unlikely that we will be able to solve the problem of malaria before we understand it, research and the development of new tools, in particular a vaccine, are now the focus of a new malaria eradication campaign that was announced by the Bill and Melinda Gates Foundation and the Rollback Malaria Partnership in 2007[7,59].

Naturally acquired immunity to malaria

Malaria infections are most severe in the very young and in pregnant women, while older children and adults in endemic areas develop immunity after multiple infections with the parasite (**Figure 3**)[60,61]. An important study by Goncalves *et al*.[62] recently showed that one episode of severe disease was sufficient to protect children against subsequent severe infections in most cases. Furthermore, while *P. falciparum* parasite densities were higher in children with severe malaria, mild high-density infections occurred before and after episodes of severe disease. The incidence of high-density infections in children remained stable across different age groups while the incidence of severe malaria decreased with age. Therefore immunity against severe disease is acquired very quickly (after one or very few episodes, **Figure 3**). Where transmission and risk of infection is high, the risk of severe disease is therefore limited to infants, young children, visitors and pregnant women, whereas in areas of low, unstable transmission almost all inhabitants are at risk of developing severe malaria[63,64]. It is hence possible that interventions that decrease the risk of infection actually increase the incidence of severe disease within a population with no previously acquired immunity[65], although this remains controversial[66,67]. Immunity against malaria is generally not sterilizing[68] and asymptomatic carrier status is the norm amongst adults in endemic areas (**Figure 3**), which ensures continuous transmission to the mosquito vector[61,69].

Naturally acquired immunity is mainly directed against blood-stage parasites and protection against pre-erythrocytic parasites does not appear to play a role in naturally exposed populations[68]. Despite decades of research there is still little direct evidence about precisely what constitutes protective immunity. It appears that early innate immune responses against blood-stage parasites shape the outcome of infection[70,71]. Naturally acquired immunity against blood-stage parasites depends to a large extent on antibodies[72,73]. Development of an effective antibody response is in turn dependent on an effective priming of the CD4 response[74]. A main risk factor for the development of severe disease appears to be an overly

inflammatory cytokine milieu[75-77]. For example severe malarial anaemia can be associated with an insufficient interleukin (IL)-10 response to high tumor necrosis factor (TNF) concentrations[78,79], but the causal relationship remains unclear.

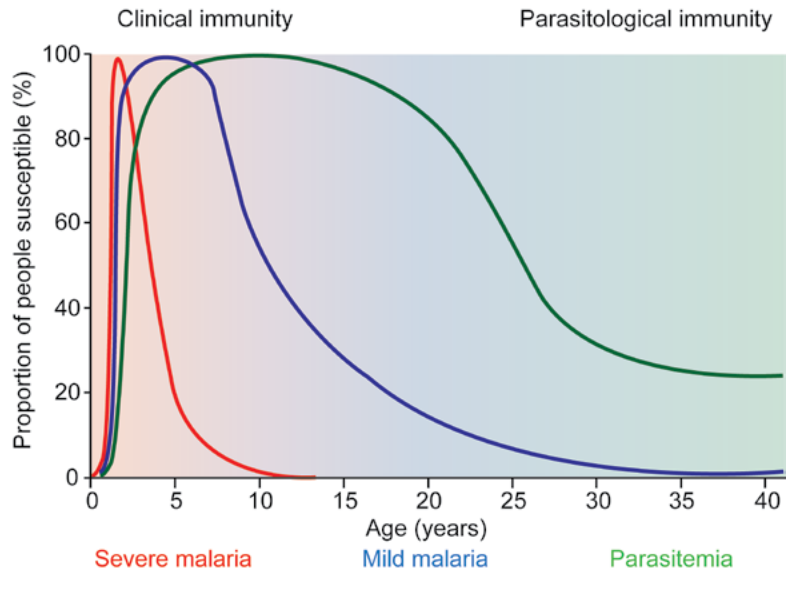

Figure 3 | Naturally acquired immunity as relation between age and malaria severity
All infants are initially susceptible to severe malaria (red line), but with repeated exposure clinical immunity is acquired by children, which makes symptoms of malaria milder (blue line), while parasites are still present in the blood-stream of almost every child. Even in adulthood many people in endemic areas harbor microscopy-detectable blood-stage parasites (green line) as parasitological immunity is very slow to develop and incomplete. The here shown timings for the development of clinical and parasitological immunity are typical for areas of moderate transmission.
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The role of humoral immunity for protection against malaria

Immunity against blood-stage malaria parasites is associated with a protective antibody response. Transfer of immunoglobulin (Ig) from clinically immune adults to children experiencing high-density parasitemias is able to reduce parasite numbers and clinical disease[72]. Furthermore immune IgG inhibits *P. falciparum* growth *in vitro*[73]. Antibodies can have a variety of antimalarial effects, the contribution of which to protection remains to be determined: i) inhibition of merozoite invasion[80]; ii) prevention of rosette formation and sequestration[81]; iii) antibody mediated cellular cytotoxicity[82]; iv) opsonisation for phagocytosis[83]; v) help for T cell effector response[84]. There are even reports suggesting that antibodies

inhibit parasite-induced inflammation, thereby controlling severe disease[85]. Despite the overwhelming evidence of the importance of antibodies for protection against blood-stage malaria infection, the identity and relative contribution of different antigenic targets remains unknown. It is likely that protection requires the induction of antibodies against multiple antigens. Kenyan children, for example, experienced no clinical disease if they had high antibody titres against 5 or more *P. falciparum* blood-stage antigens[86].

In the absence of parasite exposure the number of short-lived plasma cells producing antibodies declines, leading to a drop in circulating antibody levels[87]. Low levels of antibodies are provided by long-lived plasma cells residing in the bone marrow. The breadth and magnitude of the malaria-specific antibody response increases with age and exposure in endemic areas[88,89]. In a seasonal high transmission setting antibody responses expand during the malarious wet-season, but then contract during the 6-month malaria-free dry-season to levels just slightly higher than before transmission, leading to a gradual increase of antibody levels with cumulative parasite exposure which plateaus as adulthood is reached[89]. This is unlike antibody acquisition kinetics for other pathogens such as the measles virus, where one exposure (and the persistence of antigen) is sufficient to provide life-long levels of protective antibody[90]. It was therefore speculated that multiple infections are necessary to fill long-lived plasma cell niches with cells specific for any given malaria antigen in order to provide basal protective antibody levels against this complex parasite[89]. This extensive population of bone marrow niches with malaria specific long-lived plasma cells can dysregulate previously acquired long-lived plasma cell responses as shown for influenza in a rodent malaria model[91].

Humoral immune memory is not only mediated by long-lived plasma cells, which continue to produce antibodies, but also by quiescent memory B cells that circulate and rapidly differentiate into antibody-secreting cells upon antigen re-encounter[92]. Therefore the presence of antigen-specific memory B cells in peripheral blood is a more reliable read-out for past exposure than plasma antibody[93]. Studies in areas of very low transmission in Madagascar[94] and Thailand[95] suggest that memory B cells are maintained even in the absence of exposure for many years. The magnitude of the malaria-specific memory B cell response is similar to that induced by childhood vaccinations like Tetanus in the same population[96]. However a substantial proportion (40-70%) of naturally immune adults in endemic areas still lack detectable numbers of memory B cells specific for malaria antigens like apical membrane antigen (AMA)-1 and merozoite surface protein (MSP)-1[88,89], even in highly endemic areas where people receive an estimated 50-60 infectious bites per person per month during malaria season[97]. By contrast, a single small pox vaccination generates long-lived (>50 years) memory B cells in nearly all volunteers[98].

Because of the relatively inefficient acquisition of long-lived plasma cells and memory B cells it was suggested that humoral immunity is dysregulated during malaria infection. The Ig binding cysteine-rich interdomain region (CIDR) α of *P. falciparum* erythrocyte membrane protein (PfEMP)-1 causes polyclonal B cell activation and thus hypergammaglobulinemia in a T cell-independent manner[99]. Excessive induction of B cell activating factor (BAFF) in the host may influence B cell function and change the composition of the entire B cell compartment systemically[100,101]. Atypical memory B cells, which were originally described as “exhausted” in HIV infection, are expanded in chronic malaria infection but it is unclear whether their role is protective or detrimental for the outcome of infection[102]. Furthermore pattern recognition receptors (PRRs), which are expressed on dendritic cells and B cells can become tolerant upon chronic malaria infection, thereby downregulating the antibody and memory B cell response[103]. Malaria-naïve volunteers given a MSP-1 or AMA-1 vaccine together with the toll-like receptor (TLR) 9 agonist CpG, were shown to acquire memory B cell more rapidly, in greater numbers and maintain them for longer compared to vaccinees immunized without CpG[104]. This result did however not repeat in semi-immune adults in endemic areas[105]. All these observation could suggest a malaria-intrinsic dysregulation of the B cell response, but could equally just reflect that mounting an effective response against a pathogen with very diverse, polymorphic antigens is difficult and inefficient.

While humoral immunity is a central component of erythrocytic immunity, the contribution of antibodies to pre-erythrocytic protection (if at all observed)[68] remains controversial. However, antibodies in sera from exposed people do recognize sporozoites with a magnitude that reflects cumulative parasite exposure including transmission intensity and age[106-108]. Humans protected from malaria after immunization with infectious mosquito bites under chloroquine cover in the controlled human malaria infection model (see below) mount a functional antibody response that can inhibit sporozoite traversal *in vitro* and reduces *P. falciparum* liver parasite burden in humanized mice[109].

The contribution of rodent malaria models to our understanding of malaria

Research using *Plasmodium* species infecting rodents as models for human malaria has been indispensable for our understanding of parasite biology, genetics, evolution and ecology as well as for antimalarial immunity. The life-cycle, morphology of the different stages, metabolomic pathways and genomic organisation[110] are to a large extent conserved between human and most rodent malaria species. Four *Plasmodium* species were isolated from wild African rodents between 1943 and the 1960s (**Figure 4**, **Table 2**)[111]. *P. berghei* was discovered in the relatively cool gallery forest of Katanga (modern day Democratic Republic of Kongo) in *A. dureni* mosquitoes and in the African thicket rat *Grammomys surdaster*[112]. *P. vinckei* was originally also identified in Katanga but subspecies were later found in different parts of Africa in *Thamnomys rutilans*. The other two rodent species *P. yoelii* and *P. chabaudi* were also isolated from *Thamnomys rutilans*[113], in a more tropical climate in the Central African Republic[114], which is similar to the climate where human *Plasmodium* species thrive (**Figure 4**, **Table 2**). Infection rate of thicket rats parasitized with *P. yoelii* and *P. chabaudi* was close to 100% in this area and parasitemia persists for a long time in these rats[111]. Apart from *P. berghei*, which after initial isolation was hardly found in wild animals, subspecies of all other rodent parasites were isolated across Cameroon, Congo and Nigeria (**Figure 4**).

All isolated rodent malaria parasites can infect laboratory mice and have a short liver-stage development of just over 2 days (**Table 2**). *P. berghei* and *P. yoelii* invade reticulocytes and their asexual blood-stage replication is asynchronous, whereas *P. vinckei* and *P. chabaudi* replicate synchronously in young and mature erythrocytes (**Table 2**). The distinct species and cloned strains exhibit different infection characteristics in different mice[115,116], which can make data interpretation difficult[117]. Parasite factors together with host genetic background critically influence the pathology elicited by malaria infection. BALB/c mice are 2000 times more susceptible to *P. yoelii* compared to *P. berghei* infection, which was attributed to more effective innate immune responses that eliminate pre-erythrocytic *P. berghei* parasites[118]. Even the closely related *P. y. nigeriensis* strains 67 and 67C trigger fundamentally different innate immune responses[71]. Increased inflammation elicited by the more virulent 67C strain is responsible for severe disease and death caused by this parasite[71]. The same parasite can also have different pathogenesis in different inbred mouse strains: *P. berghei* ANKA causes lethal experimental cerebral malaria in C57BL/6 mice, severe anaemia in BALB/c mice and respiratory distress syndrome in DBA/2 mice[119].

Rodent malaria models are especially valuable to study pre-erythrocytic parasite life-cycle stages, which are not accessible in humans due to obvious ethical constraints[119]. Genetic manipulation of the *P. berghei* genome[120-122] and more recently *P. yoelii*[123] and *P. chabaudi*[124] allows labelling of parasites with fluorescent or other tags and to knock-out and over-express genes. Imaging of fluorescent or bioluminescent parasites has revealed that sporozoites are deposited into avascular parts of the dermis from where they have to actively migrate into blood vessels, which can be inhibited by antibodies[125]. Furthermore infection with bioluminescent parasites can be followed in real time through the liver- and blood-stages in a non-invasive manner by using *in vivo* imaging[126], thereby testing the effects of treatment or vaccination on parasite load.

Rodent malaria models are particularly valuable for studying immune responses to malaria in mechanistic detail. Genetically altered mice lacking key components of the immune system can contribute substantially to our understanding of antimalarial

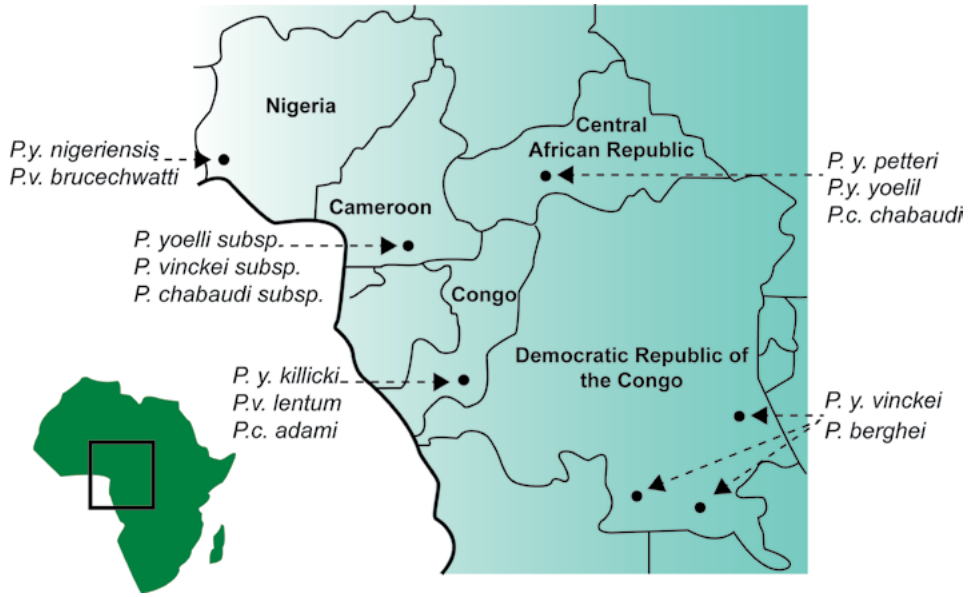

Figure 4 | Origin of rodent malaria species
All rodent *Plasmodium* species were isolated from wild *Grammomys surdaster* (*P. berghei*) or *Thamnomys rutilans* (*P. yoelii (y.), P. chabaudi (c.)* and *P. vinckei (v.)*) in central West Africa between 1943 and the mid 1960s. Diverse subspecies of all rodent *Plasmodium* parasites were found in different geographical locations, apart from *P. berghei*, which was only isolated once in the cool forests of Katanga (Democratic Republic of Kongo). The natural mosquito vector (*A. dureni*) is only known for *P. berghei* and *P. v. vinckei*.
Reproduced from The European Malaria Reagent Repository,
http://www.malariaresearch.eu/content/rodent-malaria-parasites

Table 2 | Life-cycle characteristics of rodent *Plasmodium* species[a]

Rodent parasite species	Sporogony temperature	Length of liver-stage	Erythrocyte preference	Synchronicity in blood-stage
P. berghei	19-21°C	50h	Reticulocytes	Asynchronous
P. y. yoelii	24°C	43-50h	Reticulocytes[b]	Asynchronous
P. y. killicki	22-24°C	46-50h	Reticulocytes	Asynchronous
P. y. nigeriensis	24°C	47-50h	Reticulocytes	Asynchronous
P. y. subsp	?	?	?	?
P. v. vinckei	20-21°C	53-61h	Normocytes and reticulocytes	Synchronous
P. v. lentum	24-25°C	<72h	Normocytes and reticulocytes	Synchronous
P. v. brucechwatti	24-25°C	61-65h	Normocytes and reticulocytes	Synchronous
P. v. petteri	24-26°C	53-61h	Normocytes and reticulocytes	Synchronous
P. v. subsp.	?	?	?	?
P. c. chabaudi	26°C	52-53h	Normocytes and reticulocytes	Synchronous
P. c. adami	24-26°C	?	Normocytes and reticulocytes	Synchronous
P. c. subsp.	?	?	?	?

[a] Adapted from Stephens et al. 2012[258]

[b] P.y. yoelii 17XL and YM can invade normocytes

immunity. Basic characteristics of the humoral immune response against blood-stage parasites are similar to humans e. g. passive transfer of hyperimmune serum can reduce parasitemia in *P. b. yoelii* infected mice[127] suggesting that antibodies clear blood-stage parasitemia[72]. Infection of J_HD mice[128], which are devoid of functional B cells, with *P. c. adami*, *P. c. chabaudi* CB and *P. v. petteri* resulted in clearance of the infection in the acute phase, while infecting of the same mice with *P. y. yoelii* 17X lead to death by hyper-parasitemia[129].

Despite their usefulness for malaria research it is vital to design animal models carefully to mimic the right aspects of human infections in order to draw translatable conclusions.

Controlled human malaria infection

Rodent and other animal models offer a broad range of possibilities to gain insights into the biology of *Plasmodium* infection. However it is critical to test hypothesis

gained from these models in humans. This can be done in field studies, which are however complex and therefore expensive to organize requiring a relatively large number of participants to allow for variables such as genetic heterogeneity, co-infections and (re)-infection rate. To test a growing number of new drugs and candidate vaccines in Phase IIa clinical trials and to study human antimalarial immune responses, controlled human malaria infection models have been established that mimic natural infections under strictly controlled clinical and laboratory conditions[130]. Facilitated by the *in vitro* production of gametocytes for mosquito infection[131,132], over 2500 volunteers have been exposed to *P. falciparum* infected *Anopheles spp.* mosquito bites in three specialized centres- namely the Walter Reed Army Institute of Research, Silver Spring, Maryland (USA), the University of Oxford (UK) and the Radboud University Nijmegen Medical Center (RUMC, The Netherlands)- since the 1980s. Results are reproducible between the different locations, highlighting the robustness of this model[133].

Exposure to 5 mosquitoes infected with the *P. falciparum* strain 3D7 or its parental strain NF54 ensures malaria infection in clinical trial participants of close to 100%, leading to detection of blood-stage parasites by thick blood film approximately 9-11 days later, at which point the infection is treated with antimalarial drugs[130]. Most volunteers experience non-specific symptoms of uncomplicated malaria most frequently fever and headaches. Time to patency by thick blood film is used to estimate efficacy of a given intervention and is complemented by exact measurement of blood-stage parasite burden (up to three times daily) using quantitative RealTime (qRT) PCR[134]. Pooled data from clinical trials at RUMC show that between an estimated 23 and 5273 (mean 500) merozoites per ml are released within the first erythrocytic cycle from the liver (suggesting 2-1500 infected hepatocytes) approximately 6.5 days after mosquito bite[130]. Thereafter *P. falciparum* erythrocytic replication follows a synchronous 48h pattern with blood-stage multiplication rates of approximately 10.8[130]. Because these parameters are so consistent the number of participants in each trial can be small and still provide enough statistical power to evaluate the preliminary efficacy of pre-erythrocytic vaccine candidates[130]. Since treatment is necessary after only 2-3 erythrocytic replication cycles it has been questioned whether this system is appropriate to evaluate partially effective blood-stage vaccines, which could have effects only apparent after several erythrocytic replication cycles, reducing morbidity and mortality[135].

Therefore a blood-stage challenge model was developed which utilizes cryopreserved parasitized blood from two volunteers experimentally infected with

the 3D7 strain of *P. falciparum* by mosquito bite[136]. Injection of 1800 parasitized erythrocytes *iv* leads to blood-stage parasitemia that can be detected by qRT PCR after 3.5 days and reaches thick blood-film detectable levels after 8 days, thus allowing more time to detect effects targeting blood-stage parasites[135]. This approach may however miss potential cross-protective immune responses, which could enhance vaccine efficacy by protecting against pre-erythrocytic parasites.
As the company Sanaria Inc. can produce aseptically purified, cryopreserved sporozoites[137], these can be used instead of mosquito bites to experimentally infect humans. Here, the number of sporozoites injected can be controlled thus minimizing inter-individual variations, and statistical power is improved, compared to infections initiated by mosquito bite. Furthermore cryopreserved sporozoites make it possible to conduct trials all over the globe as no access to highly specialized *P. falciparum* insectaries is required. It was shown in malaria naïve adults in the Netherlands[138] as well as in adult Tanzanians from an endemic area[139] that *intradermal* (*id*) injection of 10,000 or 25,000 cryopreserved sporozoites is safe, well tolerated and infectious for a large proportion of individuals. Microscopic patency is substantially delayed by over 6 days compared to an infection with 5 mosquito bites suggesting that *id* injection is very ineffective with few sporozoites actually reaching the liver to establish an infection[140].
Together these controlled human malaria infection models are powerful tools to test drugs and candidate vaccines in small clinical trials before attempting large-scale field trials.

Malaria vaccine development

Recent advances in malaria research have lead to a new enthusiasm to eradicate malaria[59]. It is now recognized that given the complexity of the problem no single strategy will suffice. However, the only successful eradication programme so far (smallpox) relied on a effective vaccine; something that is lacking for malaria to this date[141]. Successful vaccines have been developed for viral and bacterial diseases, against which immunity is acquired after a single infection by the induction of high levels of protective antibodies. It has proven to be much more challenging to develop a vaccine against diseases that do not naturally induce sterile immunity like HIV, *Mycobacterium tuberculosis* and complex eukaryotic pathogens including *Plasmodium*[142]. However clinical immunity against blood-stage malaria parasites, which does not necessarily clear parasitemia, can be acquired naturally with repeated exposure (**Figure 3**), therefore it should be

feasible to develop a malaria vaccine. A vaccine could either mimic naturally acquired immunity and target blood-stage parasites, reducing severe clinical disease, or target pre-erythrocytic stages, which, if 100% effective, would not only preclude all symptoms of malaria but also block transmission. Another advantage of targeting pre-erythrocytic parasite life-cycle stages is their small number (**Figure 2**) and that it takes several days for human *Plasmodium* parasites to complete liver-stage development[143]. If however even a single infected hepatocyte escapes the immune response, the resulting blood-stage infection (release of an estimated 10,000 merozoites from each infected liver-cell and exponential multiplication, **Figure 2**) would cause severe symptoms if no erythrocytic immunity was previously acquired. In fact mathematical modelling showed that even a 99% effective pre-erythrocytic vaccine leads only to a delay of two multiplication cycles until pathogenic blood-stage parasite densities are reached[144] (**Figure 5A**). On the other hand even an only 40% effective blood-stage vaccine lets blood-stage parasitemia increase slower, while a 90% reduction of blood-stage parasitemia in each cycle actually overrides the blood-stage multiplication effect and leads to a decrease in parasitemia over time and eventual clearance[144] (**Figure 5B**). Both blood-stage and pre-erythrocytic vaccine strategies utilizing individual parasite proteins or whole attenuated parasites are pursued, as summarized in the WHO rainbow table[145,146]. It is however likely that both strategies have to be combined in order to develop an effective multi-stage malaria vaccine. A 70% reduction in liver parasite burden combined with a 90% effective blood-stage vaccine would clear parasitemia eventually and keep parasite densities under 50 parasites per ml, which is 10-fold below pathogenic blood-stage parasite densities[144] (**Figure 5C**). Additionally vaccines that block transmission, which have only delayed benefits for the vaccinee, could be incorporated[147]. Since a multi-stage vaccine is still lacking, blood-stage and pre-erythrocytic vaccination approaches using either individual proteins (subunit vaccines) or whole parasites are introduced separately below.

Blood-stage antigens as subunit vaccine

Using individual blood-stage malaria antigens for immunization was thought to be able to direct antimalarial immunity to few epitopes thereby maximising efficiency while minimizing unwanted side-effects[148]. As passive transfer of immunoglobulin from naturally immune adults was shown to protect vulnerable children[72,149], antigens that were recognized by serum from clinically immune individuals were selected[150,151]. Therefore only few immunodominant, but also highly polymorphic, antigens were carried forward and assessed in Phase I and IIa clinical trials- with

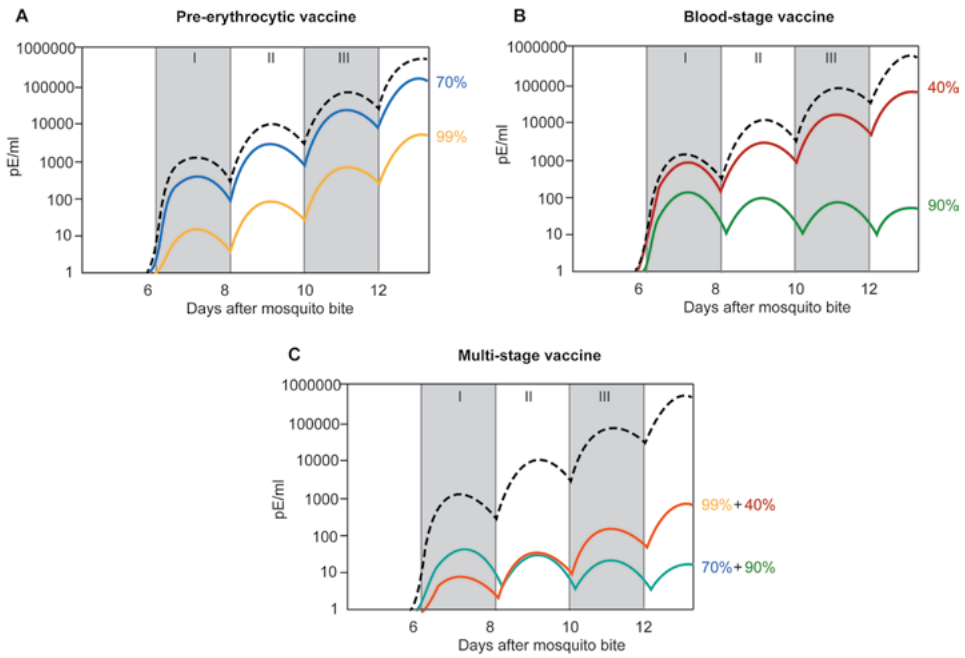

Figure 5 | Effect of pre-erythrocytic, blood-stage and multi-stage vaccines on blood-stage parasite densities

Simulated erythrocytic parasite densities (in parasitized erythrocytes (pE) per ml) after merozoites release from 200 infected hepatocytes 6.8 days after *P. falciparum* mosquito bite infection. Exponential replication within the first 3.5 erythrocytic cycles (Roman numerals) is shown with the black dotted line. **A)** Effect of a pre-erythrocytic vaccine that kills 70% (blue) or 99% (yellow) of all infected hepatocytes. **B)** Effect of 40% (red) and 90% (green) effective blood-stage vaccine. **C)** Combined effect of a multi-stage malaria vaccine with 99% pre-erythrocytic efficacy plus 40% efficacy against blood-stage parasites (orange) or 70% pre-erythrocytic and 90% blood-stage vaccine effect (turquois).
Based on figure by Hermsen *et al*., 2004[144], reproduced with permission by the publisher

very limited success (reviewed[148,152]). One of the first malaria vaccines tested on a large scale was S*Pf*66 which consisted of three merozoite-derived epitopes linked together with the repeat regions of the pre-erythrocytic antigen circumsporozoite protein (CSP). Following enthusiasm after field trials in South America where it showed over 80% efficacy[153] S*Pf*66 failed to induce any protection in Africa or Thailand. After a Cochrane review it was decided that there was no justification to continue clinical trials using this vaccine formulation[154]. Combination B, consisting of MSP-1 and -2 as well as ring-infected erythrocyte surface antigen (RESA), was reported to be safe, but only sub-optimally immunogenic[155,156]. Given that 5300 genes are encoded in the *P. falciparum* genome[157], it seems likely that there are many other proteins, which have not been investigated to date, that could mediate protection. In a recent study, Kenyan children mounted antibody responses

against novel and little studied natively folded merozoites surface or secreted full-length ectodomain proteins[158]. Protective efficacy of these antibody responses, as assessed by their correlation with absence of clinical episodes, showed that some of the new proteins had the same or higher efficacy than leading blood-stage vaccine candidates like MSP-2. Certain combinations of antibody responses were associated with high levels of protection and no clinical episodes were detected if at least 5 out of 10 leading proteins were recognized[158]. Thus the identification of new protective antigens and combination of these into a multi-component vaccine inducing high antibody titres could lead to an eventual success in subunit blood-stage vaccine development.

Pre-erythrocytic antigens as subunit vaccine

Subunit vaccines based on proteins expressed during the pre-erythrocytic stages can elicit sterile immunity against sporozoites or liver-stage parasites thereby preventing generation of asexual blood-stage parasites responsible for morbidity and mortality as well as gametocytes responsible for transmission. The most advanced pre-erythrocytic subunit vaccine is RTS,S, which comprises of the C-terminal end of CSP fused to the hepatitis B virus antigen S and is administered with the adjuvant AS01 or AS02. CSP covers the entire surface of sporozoites and is an integral part of its membrane but is also found in the plasma membrane of liver-stage parasites[159]. In Phase IIa trials RTS,S was able to mediate over 50% sterile protection against controlled human malaria infection in malaria-naïve volunteers[160]. A large, multi-centre Phase III clinical trial in seven African countries with different transmission intensities (namely Burkina Faso, Gabon, Ghana, Kenya, Malawi, Mozambique, and the United Republic of Tanzania) involving 15,460 infants and young children RTS,S protected 30-50% of children against clinical and severe malaria[161-163]. Efficacy was lower in infants 6-14 weeks of age than in children aged 5-17 months and protection waned quickly within 18 months[163]. Protection was associated with the induction of high CSP-specific antibody titres and CD4 T cell responses[160,164,165]. The benefits of this moderately effective vaccine for public health are disputed[166-169]. It is surprising that RTS,S, designed to protect against pre-erythrocytic parasites, actually reduces the risk of clinical disease associated with blood-stage parasites, instead of inducing sterile protection[170]. One explanation is that a profound reduction of infected hepatocyte numbers leads to low numbers of merozoites being released into the blood-stream, which allows more time to activate adaptive immune responses before pathogenic erythrocytic parasite densities are reached. GlaxoSmithKline, who

developed RTS,S in partnership with WRAIR and PATH Malaria Initiative (Bill and Melinda Gates Foundation) have announced in July 2014 to apply for licensure of the vaccine. If successful, RTS,S will be the first protozoan vaccine ever licensed for human use.

Another pre-erythrocytic antigen, Thrombospondin related anonymous protein (TRAP) fused to the T cell multiple epitope string (ME), formulated in a heterologous prime-boost regimen with replication-deficient viral vectors[171], was shown to elicit very high protective CD8 T cell responses in malaria naïve volunteers and sterilely protects 21% against mosquito bite challenge infection, whilst delaying patency in 36% of volunteers[172]. Delay in time to patency was attributed to a strong reduction of liver-parasite burden in these volunteers. Two recent Phase Ib studies in malaria-endemic areas of Kenya and The Gambia show that immunization with the chimpanzee adenovirus serotype 63 (ChAd63) and boost with modified vaccinia virus Ankara (MVA)- both encoding ME-TRAP- can induce cellular and humoral responses in previously exposed adults[173] and will now be assessed for efficacy. A combination of RTS,S with TRAP did not improve protection or delay parasitemia[174], compared to infection controls, while RTS,S alone was previously shown to induce 50% sterile protection in a similar experimental setting[160]. Thus the addition of TRAP to RTS,S has a detrimental rather than beneficial effect for the development of pre-erythrocytic immunity.

Liver-stage antigen (LSA) 1 and 3[175] have been identified as key antigens of liver-stage parasites. Formulated with AS01 and AS02, LSA 1 was safe and induced high antibody titres and CD4 T cell responses, but did not confer any protection in controlled human malaria infection trials[176]. LSA 3 can protect against *P. falciparum* challenge in chimpanzees[177] and *Aotus* monkeys[178] and was recently assessed for safety and immunogenicity in a human clinical trial (NCT00509158)[179].

Rationale for whole parasite vaccination

Identification and selection of protective parasite antigens for subunit vaccine development is difficult and has so far not lead to the desired outcome- an effective malaria vaccine. There are however vaccines that induce sterile protection against veterinarian parasitic diseases like *Theilaria*, which are based on the inoculation of whole live-attenuated or drug-treated parasites[180]. Also it has been known for centuries that humans can be immunized by inoculation of infectious *Leishmania* parasites into the skin[181]. The resulting self-limiting lesion protects against subsequent infections, preventing disfiguring lesions and serious complications like visceral leishmaniasis[182]. It therefore appears that exposure of the immune system to the attenuated parasites and therefore a broad range of antigens allows for the development of high levels of protection.

Whole blood-stage parasite immunization strategies

For malaria the first demonstration that live parasites can induce immunity came from immunization with killed *Plasmodium* blood-stage parasites and strong adjuvants, which partially protects birds, mice and non-human primates[183-187]. Low doses of 1000 *P. chabaudi* erythrocytic parasites killed by freeze/thawing combined with the adjuvant CpG-ODN, which induces potent Th1 responses, protects mice partially from challenge infection[188]. Since the need for strong adjuvants precludes the use of killed blood-stage parasites as an immunization strategy in humans, an alternative approach uses live, irradiated blood-stage parasites, which are incapable of further erythrocytic replication. Immunization with large numbers elicits partial protection against blood-stage challenge in chicken[189,190], as well as in rats, mice[191-193] and monkeys[194]. Blood-stage parasites can also be attenuated using centanamycin[195], which leads to the persistence of parasite DNA for 110 days and induces strong species-transcending protection in the mice[196].

Moreover, targeted gene deletion can arrest *P. falciparum* blood-stage parasites in culture[197-200] and immunization with genetically attenuated blood-stage parasites protects mice against infectious challenge[201-204]. Purine metabolism especially, which is unique to *Plasmodium*, is an attractive target for directed genetic manipulation[203,204]. It is important to note that gene knock-out needs to be conditional as parasites could otherwise not be manufactured for vaccine development.

Treatment of blood-stage infections before pathogenic parasite densities are reached also elicits protection against re-infection. *P. yoelii* blood-stage parasites combined with chloroquine treatment curtailing the infection can reduce liver parasite burden after challenge with 35,000 sporozoites *iv*, which is the first direct evidence of cross-stage immunity[205]. Immunization with *P. yoelii* blood-stage parasites under mefloquine cover did not lower liver parasite burden after sporozoite challenge but attenuated blood-stage parasite growth even after challenge with a different species (*P. vinckei*)[206]. Inoculation of 100,000 *P. chabaudi* parasitized erythrocytes followed by atovaquone/proguanil treatment protected mice against homologous and heterologous blood-stage challenge[207]. This vaccination strategy is also the only whole blood-stage parasite immunization strategy ever tested in humans: Volunteers were immunized with multiple ultra-low doses of approximately 30 infected erythrocytes, followed by treatment with atovaquone/

proguanil. These people were protected against subsequent challenge with the same blood-stage inoculum[208]. Results may however have been confounded by persisting atovaquone-levels[209]. Immunity was associated with CD4 and CD8 T cells producing interferon (IFN) γ and nitric oxide, but not with antibodies binding parasitized erythrocytes. It therefore appears that induction of cellular immune responses is important for the generation of effective blood-stage immunity. Thus selection of antigens for blood-stage subunit vaccine development may have missed promising candidates, since it is based on antigens that are recognized by antibodies from immune individuals. Immunization with drug-, chemically- and genetically-attenuated blood-stage parasites hence has the potential to protect against malaria and should be investigated further: either as whole parasite vaccination strategy or to identify new protective antigens.

Pre-erythrocytic whole parasite immunization strategies

As for blood-stage parasites, irradiation (or UV-inactivation) of sporozoites causes such extensive DNA damage that they are incapable of further replication. However irradiated sporozoites can still invade hepatocytes and develop into uni-nucleated trophozoites, but never establish a blood-stage infection. Richards *et al*. demonstrated in 1966 that UV inactivation of *P. gallinaceum* sporozoites protects chickens against subsequent infections[190]. High numbers of irradiated *P. berghei* sporozoites were thereafter shown to sterilely protect susceptible A/J mice from lethal sporozoite challenge[210], but not from direct blood-challenge[211], which established the paradigm that immunity to malaria is stage-specific. These experiments were the first to validate that sterile protection against a parasitic disease is feasible and sparked much excitement. In 1973 one volunteer was immunized with over 1000 *P. falciparum* infected mosquito bites over the course of one year and challenged with infectious bites against which he was protected[212]. A direct transfer of blood-stage parasites however lead to patent blood-stage parasitemia that had to be treated, so again immunization with irradiated sporozoites did not seem to induce erythrocytic immunity, although blood-stage parasite growth rates and time to patency could not be compared to a naïve volunteer[212]. Immunity elicited by irradiated sporozoite immunization was strain-transcending[212], but partially species-specific[213]. It was initially believed that the main mechanism of protection was the inhibition of sporozoite invasion by antibodies. CSP was identified as prominent antigen and used to formulate RTS,S[214,215] (see above), but many antigens with potential protective properties remain to be investigated. A protein-microarray study identified three

novel antigens that were recognized by protected volunteers immunized with irradiated sporozoites (and not unprotected controls), but at a lower magnitude than CSP or TRAP[216]. Despite the superior sterile immunity induced by this whole parasite immunization with irradiated sporozoites it was not deemed practically feasible to turn it into a commercial vaccine. However, Sanaria Inc. addressed some of these obstacles and can now produce aseptically dissected, purified and cryopreserved irradiated sporozoites. Immunization of volunteers with these irradiated sporozoites *id* and *subcutaneously* was safe but sub-optimally immunogenic[217], while 5 immunizations with 135,000 sporozoites *iv* protected against mosquito bite challenge 5 months later[218]. Currently the efficacy of this vaccine is evaluated in adults in malaria-endemic areas of Mali. Sterile protection induced by immunization with irradiated sporozoites is strongly associated with the induction of high frequencies of CD8 T cells[219,220] and persistence of parasite antigen in liver, draining lymphnodes and spleen[221].

Similar to irradiation, treatment of sporozoites with centanamycin arrests parasite development in hepatocytes[222] and repeated immunization with high doses elicits species-transcending protection against sporozoite challenge in mice[223].Disruption of genes essential for the completion of liver-stage development[224-230] equally halts progression through the life-cycle in pre-erythrocytic stages and can lead to the induction of pre-erythrocytic immunity, which is CD8 T cell-dependent[231]. This immunization strategy was also tested in a first trial in humans using *p52*- and *p36*-deficient *P. falciparum* infected mosquito bites[232]. Following exposure to 263 bites one volunteer developed blood-stage parasitemia, which was confirmed to be caused by the transgenic parasite[232]. Blood break-through infections are thus a major concern when pursuing genetically attenuated parasites as a malaria vaccine[233].

It was argued that arrest late in liver-stage development can induce more powerful protection[234]. This may be because antigenic targets become increasingly similar to blood-stage parasites[235], which can allow for the development of cross-stage immunity.

Chemoprophylaxis and sporozoite immunization

Other than the stage-specific experimental vaccination approaches described above, chemoprophylaxis and sporozoite (CPS) immunization allows exposure to both pre-erythrocytic and blood-stage parasites. It was first described in rodents that injection of *P. berghei* sporozoites combined with prophylactic chloroquine

treatment induces protection against sporozoite challenge[236-238]. Chloroquine inhibits the conversion of toxic free heme into hemozoin, which leads to the lysis of the parasitized erythrocyte[239]. Pre-erythrocytic immunity induced by CPS immunization, i.e. the reduction of liver parasite burden, was first confirmed after sporozoite challenge of mice immunized by *iv* injection of *P. yoelii* sporozoites, while receiving chloroquine[240]. Rodent studies have thereafter concentrated on substituting chloroquine for other antimalarial drugs[206,241,242] and on delineating the mechanism of pre-erythrocytic immunity[243-245], which appears to be dependent on the induction of large numbers of CD8 T cells producing IFNγ[243]. However immunity against blood-stage parasites can also be elicited by this immunization regimen, especially if exposure to erythrocytic parasites is prolonged e.g. when subcurative chloroquine regimens are used[206,242,244]. CPS-induced blood-stage immunity can be dependent on the induction of antibodies[244]. Immunization with *P. yoelii* and mefloquine can protect against *P. vinckei* sporozoite- and (to a lesser extend) blood-challenge[206], thereby showing that protection following CPS immunization is species-transcending.

In humans 12 to 15 bites of mosquito infected with the NF54 strain of *P. falciparum* and prophylactic chloroquine treatment elicits sterile immunity against mosquito bite challenge[246]. Re-challenge of previously sterilely immune volunteers 2.5 years after the last immunization revealed that protection is long lasting (although potentially dependent on boosting by first challenge infection), with four of six volunteers still sterilely protected[247]. The remaining two volunteers had a significantly delayed onset of patent parasitemia compared to infection controls[247]. All protected volunteers mounted pluripotent effector memory T cell responses producing IFNγ, TNF and IL-2[246], which were stronger after re-stimulation *in vitro* with blood-stage parasites rather than sporozoites[247]. A dose de-escalation trial where immunization dose was reduced to 10 or 5 infective bites instead of 15 given three times also showed a strong association between the magnitude of T cells expressing cytotoxic markers (CD4 T cells expressing CD107a and CD8 T cells expressing Granzyme B) and sterile protection from mosquito bite challenge infection[248]. In this thesis we address whether antibody and memory B cell responses against a set of immunodominant malarial antigens associate with protection from mosquito bite challenge infection. Only half of the volunteers immunized with three doses of 5 mosquito bites were sterilely protected, while the others developed thick blood film detectable parasitemia before day 21 and had to be treated. Patency was delayed by a median of 2.5 days compared to infection controls, but the delay was not significant[248]. Therefore the development

of protective immunity following CPS immunization is dependent on the number or immunizing mosquito bites.

The complete absence of blood-stage parasites detectable by qRT PCR following mosquito bite challenge, led to the assumption that CPS-induced protection in humans is directed against pre-erythrocytic parasites. To address the question of stage-specificity, volunteers were immunized three times with 8 *P. falciparum* NF54 and 7 *P. falciparum* 3D7 infected mosquito bites and challenged with *P. falciparum* 3D7 infected erythrocytes[136] by *iv* injection[249]. Patency and blood-stage multiplication rates were not different between immunized volunteers and infection controls after direct blood challenge, suggesting that all immunity is directed against pre-erythrocytic parasites.

Since millions of travellers take chemoprophylaxis every year and intervention programmes using mass drug administration or intermittent preventive treatment of risk groups such as schoolchildren are employed in malaria endemic areas[250], there should be evidence of protection induced by chemoprophylaxis and repeated exposure to infected mosquito bites. Despite worries that the incidence of disease and mortality after termination of drug treatment might rise (rebound effect), treatment with sulfadoxine-pyrimethamine was shown to protect infants and children against clinical malaria well beyond the pharmacological effects of the drug[251], suggesting that chemoprophylaxis can positively enhance natural immunity. Unfortunately the massive methological differences between studies preclude unequivocal conclusions about the effect of chemoprophylaxis on naturally acquired immunity[252].

CPS immunization is an invaluable tool to address fundamental questions about the contribution of each life-cycle stage to protection against malaria and the immune mechanisms involved. It can be used to identify new antigenic targets and immune-correlates of protection from both pre-erythrocytic and blood-stage parasites. Given its demonstrated potency, initiatives by Sanaria Inc. and partners are ongoing to explore its potential application as vaccine (PfSpzCVac) for special groups such as military personnel and/or tourists.

Rationale for choosing the *P. chabaudi* model to study the stage-specificity of protection and the role of humoral immunity after CPS immunization

An important question is which parasite life-cycle stages are the source and target of antimalarial immunity after CPS immunization. In this thesis we develop a novel mouse model of CPS immunization, resembling human clinical trials in

many aspects, to address the life-cycle stage specificity of protection. These studies have to be undertaken in rodent models since obvious ethical constraints preclude analysis of liver parasite burden in human volunteers, which is the only direct evidence for pre-erythrocytic immunity[206,240,241,245]. Furthermore blood-stage infection can be examined beyond patency, which may allow the detection of partial blood-stage immunity[135]. In humans only two erythrocytic cycles could be monitored by qRT PCR before thick blood film patency is reached (see above).

We CPS immunize C57BL/6 mice with two different clones of *P. c. chabaudi* (AS and CB, hereafter referred to as *P. chabaudi*) with different virulence phenotypes[253]. *P. chabaudi* was chosen since it has a synchronous life-cycle and is the only rodent parasite that mostly establishes chronic, recrudescing blood-stage infections in mice. Furthermore, it exhibits many characteristics associated with the pathogenesis of human malaria, such as rosetting[254], sequestration[255] and antigenic variation[256,257]. *P. chabaudi* is therefore an ideal model to study parasite-host interactions in an *in vivo* setting and has critically contributed to our understanding of antimalarial blood-stage immunity[258]. Distinct cloned lines of *P. chabaudi* have been extensively phenotypically characterised and genome sequencing revealed that they are also genetically very polymorphic, displaying distinct virulence phenotypes[253,257] [Otto T. D. *et al*., BMC Biology in press.

Until recently most *P. chabaudi* parasites were maintained by serial blood passage of infected erythrocytes from one mouse to a naïve recipient, which was shown to enhance parasite virulence[259]. A recently described optimized protocol of laboratory mosquito transmission of *P. chabaudi*[260] allows us now to study this parasite in the context of the full life-cycle and CPS immunization to be conducted by mosquito bite like in human volunteers. Infections with *P. chabaudi* by mosquito bite can persist for up to 100 days in mice[259], but is even more chronic in its natural host *Thamnomys rutilans*[113]. Chronic infections are typical for untreated infections with human malaria parasites[61]. Of special importance to us are the similarities of protection against re-infection between *P. chabaudi* and *P. falciparum*. Neurosyphilis patients receiving malaria-therapy, which was considered standard medical care at the beginning of the 20th century, showed clinical and parasitological immunity after reinfection with a homologous (and to a lesser degree heterologous) *P. falciparum* strain, which was evident by few parasites in circulation and less, lower intensity fever episodes[261]. Equally one self-cured infection with *P. chabaudi* by mosquito bite elicits strain-transcending immunity against homologous and heterologous re-challenge, reducing the number of mice developing patent infections and drastically reducing peak parasitemia in the ones that do[259]. Infections with serially blood-passaged *P. chabaudi* parasites seem to induce protection less efficiently than mosquito transmitted parasites, but can still elicit strain-specific immunity to re-infection, whereas only limited protection against challenge with a different parasite species was observed[253,262].

Of course there are also inherent differences between *P. chabaudi* in mice and *P. falciparum* in humans. The liver-stage cycle is much shorter (52h for *P. chabaudi* instead of 158h for *P. falciparum*) and blood-stage parasitemia reaches higher levels before symptoms are observed, especially if serially blood-passaged *P. chabaudi* parasites are used[258]. Instead of fever mice develop hypothermia and the organs where *P. chabaudi* sequesters (liver and lungs) are more similar to *P. vivax* than *P. falciparum* infection[255]. However being aware of these limitations, *P. chabaudi* is in our eyes still the most appropriate model to study the stage-specificity of CPS-induced protection.

We also use *P. chabaudi* to address if antimalarial antibodies are of functional relevance for protection after CPS. The humoral response to *P. chabaudi* blood-stage infection is well characterised: Antibodies against *P. chabaudi* AS blood-stage parasites increase over the course of blood stage infection and reach high levels 30 to 80 days after mosquito transmission (**Figure 6A**)[259]. A single infection with *P. chabaudi* can give rise to short-lived and long-lived plasma cells and memory B cells[263]. Memory B cells are in circulation at all times and thus peripheral blood frequencies, as sampled in humans, are representative of their frequency in lymphoid organs[264]. Mice lacking the transmembrane exon of the Ig µ chain, that cannot produce mature B cells and thus antibodies (so called µMT mice)[265], infected with serially blood-passaged[266-268] or mosquito transmitted *P. chabaudi* (**Figure 6B**) are able to control parasitemia in the acute phase, but importantly, do not clear blood-stage parasites in the chronic phase (**Figure 6B**). Therefore it seems antibody-independent immune responses (innate immune responses and T cells) can control blood-sage parasitemia during the acute phase[129,269], while antibodies are crucial for complete parasite clearance in the chronic phase. The contribution of humoral immunity to pre-erythrocytic protection against *P. chabaudi* is unknown, since *P. chabaudi* could rarely be studied in the context of mosquito-transmission and sensitive measurements for liver parasite burden were lacking.

Taken together the new *P. chabaudi* mouse model of CPS immunization described in this thesis, which uses immunization by mosquito bite, is perfectly suited to establish the functional contribution of humoral immunity to protection and to answer fundamental questions about the stage-specificity of immunity to malaria.

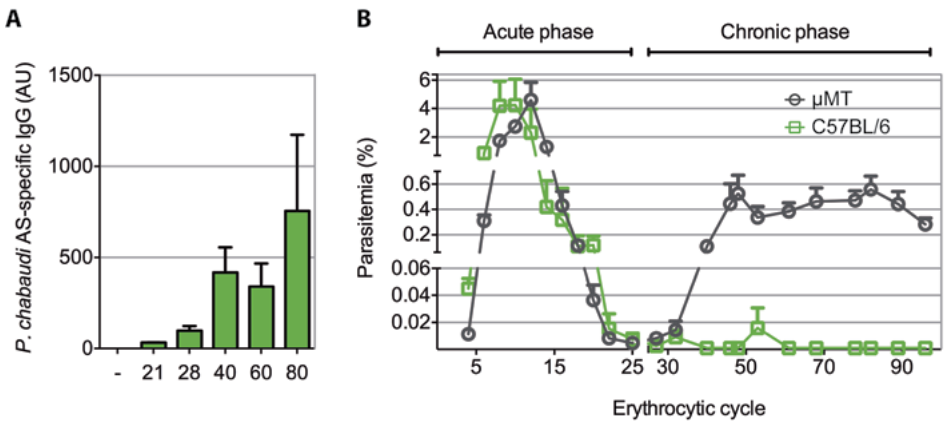

Figure 6 | A functional B cell response is indispensable for chronic parasite clearance
A) IgG plasma-antibody specific for *P. c. chabaudi* AS blood-stage parasite lysate as determined by enzyme-linked immunoabsorption assay after a primary infection of C57BL/6 mice with *P. c. chabaudi* AS by mosquito bite (n=8, displayed as arbitrary units (AU) relative to hyperimmune plasma). **B)** μMT mice (n=13), which cannot produce mature B cells and thus antibodies, and C57BL/6 (n=6) mice were infected with 10^5 *P. c. chabaudi* AS parasitized erythrocytes derived from a donor mouse that was infected by mosquito bite. The percentage of infected erythrocytes in the blood (parasitemia) was monitored for 96 days by thin blood films, which were examined by microscopy. Limit of detection was 1 parasite in 10^4 erythrocytes (0.01% parasitemia).
A reproduced with permission by the publisher from Spence *et al*., 2013[259]

Scope of this thesis

In order to design an effective malaria vaccine we have to understand what constitute protective antimalarial immunity. Immunity is thought to be *Plasmodium* life-cycle stage specific, but shared antigens between liver and blood-stage parasites[235] may mediate cross-stage immunity. Cross-stage immunity could explain why chemoprophylaxis and sporozoite (CPS) immunization protects humans against malaria with unprecedented efficacy, since chloroquine treatment allows exposure to both pre-erythrocytic and blood-stage parasites. In this thesis we aim to characterise source and target of CPS-induced immunity. We therefore design a new experimental rodent *P. chabaudi* CPS immunization model to investigate liver parasite burden and patent blood-stage parasitemia, which is not possible in CPS immunized human volunteers. *P. chabaudi* shows many crucial similarities with *P. falciparum* infections in particular with regard to protection against re-infection. Immunization and challenge regimen in this rodent model were devised to mimic infection dynamics from human CPS clinical trials including immunization by mosquito bite, which is unique among all published rodent models of CPS immunization[206,236-238,240-244] and was only recently made possible by an improved protocol for *P. chabaudi* mosquito transmission[260].
Blood samples from CPS immunized volunteers offer the unique opportunity to study the development of immunity in a setting of complete protection against the malaria parasite. Antibodies are crucial for protection against blood-stage parasites[72], but their contribution to sterile pre-erythrocytic immunity is less well defined[109]. The generation of a memory B cell response after CPS immunization of humans may also indicate whether humoral immune memory is really impaired following malaria infection, as previously suggested[100]. To analyze the generation of malaria antigen-specific memory B cells we use ELISpot analysis, which relies on an unspecific, polyclonal activation step. It is unclear if the number of antigen-specific memory B cell measured by ELISpot reflects memory B cell frequencies *ex vivo*[96,270].

Our specific objectives are therefore:

- To identify evidence for cross-stage immunity following whole parasite vaccination approaches.
- To address under which conditions pre-erythrocytic, blood-stage or cross-stage immunity are elicited following CPS immunization using a novel *P. chabaudi* mouse model.

- To answer whether humoral immunity contributes to protection after CPS immunization in both the *P. chabaudi* mouse model and in human volunteers.
- To monitor the expansion of IgG+ memory B cells during mitogen culture to validate if it is constant and thus if malaria antigen-specific cells quantified by ELISpot reflect frequencies of memory B cells *ex vivo.*

In **chapter 2** we critically evaluate published literature for evidence of cross-stage immunity from whole parasite pre-erythrocytic and blood-stage vaccination approaches. The rodent *P. chabaudi* CPS immunization model, which reflects human clinical trials in many crucial aspects, is used in **chapter 3** to answer fundamental questions about the stage- and strain-specificity of CPS-induced immunity. Using transgenic mice lacking B cells and thus antibodies, we further address if humoral immunity contributes to protection following CPS immunization. Testing responses against specific parasite antigens in peripheral blood samples from CPS immunized human volunteers in **chapter 4** extends investigations into the contribution of humoral immunity to CPS-induced protection. The acquisition kinetic and association with protection of antibody and memory B cell responses against well characterized, immunodominant pre-erythrocytic, cross-stage and blood-stage malaria antigens is studied over the course of the immunization and after different challenge regimens. Since the memory B cell ELISpot assay is used to evaluate human humoral responses after CPS immunization, we test in **chapter 5** whether antibody-secreting cells measured by ELISpot, after an unspecific, polyclonal activation step, reflect memory B cell frequencies *ex vivo* by using flow cytometry-based analysis of B cell frequencies before and after mitogen stimulation.

Our findings are discussed in a broader context with regard to their implications for future malaria vaccine development and how to design appropriate rodent models of CPS immunization in **chapter 6** and summarized in English, Dutch and German in **chapter 7**.

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CHAPTER 2

Cross-stage immunity for malaria vaccine development

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Abstract

A vaccine against malaria is urgently needed for control and eventual eradication. Different approaches are pursued to induce either sterile immunity directed against pre-erythrocytic parasites or to mimic naturally acquired immunity by controlling blood-stage parasite densities and disease severity. Pre-erythrocytic and blood-stage malaria vaccines are often seen as opposing tactics, but it is likely that they have to be combined into a multi-stage malaria vaccine to be optimally safe and effective.

Since many antigenic targets are shared between liver- and blood-stage parasites, malaria vaccines have the potential to elicit cross-stage protection with immunity against both stages complementing and enhancing each other. Here we discuss evidence from pre-erythrocytic and blood-stage whole parasite immunization approaches that show that protection against malaria is not necessarily stage-specific. Parasites arresting at late liver-stages especially, can induce powerful blood-stage immunity, and similarly exposure to blood-stage parasites can afford pre-erythrocytic immunity.

The incorporation of a blood-stage component into a multi-stage malaria vaccine would hence not only combat breakthrough infections in the blood should the pre-erythrocytic component fail to induce sterile protection, but would also actively enhance the pre-erythrocytic potency of this vaccine. We therefore advocate that future studies should concentrate on the identification of cross-stage protective malaria antigens, which can empower multi-stage malaria vaccine development.

Background

Malaria remains a major global health scourge and there is a general consensus that elimination and eradication efforts will not be successful without an effective malaria vaccine. In malaria endemic areas, immunity against severe disease caused by blood-stage parasites can be acquired after only one or two infections, while infections with high parasite densities still occur[1]. Vaccines targeting blood-stage parasites should equally induce control of (severe) disease but ultimately also clearance of blood-stage parasites. This is essential as gametocytes form during blood-stage infection and transmission to mosquitoes can thus continue.

There is only very limited evidence for protection against pre-erythrocytic malaria parasites (sporozoites and liver-stage parasites) in naturally exposed populations[2-4]. Pre-erythrocytic vaccines aim to outperform naturally acquired immunity by targeting the clinically silent stages of infection thus precluding any parasites reaching the blood stream. This would abolish any symptoms of malaria and additionally block transmission. The risk of such an approach is however that breakthrough blood-stage infections can cause severe complications, if the pre-erythrocytic vaccine is only partially effective. Therefore a blood-stage component should be included to minimize this risk[5,6]. This is especially important since it was suggested that declining transmission intensity and thus reduced boosting of clinically protective blood-stage immunity could in fact increase overall malaria morbidity[7]. The desired scenario would therefore be to develop a multi-stage malaria vaccine that minimizes both transmission and disease[8].

Hypothesis: Shared antigenic targets between liver and blood-stage parasites can induce cross-stage immunity

Given that there are shared antigens between the different life-cycle stages of the malaria parasite[9], it is possible that functional immunity to pre-erythrocytic and blood-stage parasites could enhance each other, offering an intriguing possibility for development of a multi-stage malaria vaccine. Evidence for cross-stage immunity comes from several studies[10-13]. For instance, apical membrane antigen (AMA)-1 and merozoite surface protein (MSP)-1 are highly abundant in blood-stage parasites with roles in erythrocyte invasion[14,15]; however these antigens are also expressed by sporozoites and liver-stage parasites[16,17]. In human volunteers immunized with AMA-1 the number of parasitized erythrocytes during the first blood-stage cycle after mosquito bite challenge infection was about 7-times lower compared to non-immunized controls, suggesting that pre-erythrocytic immune responses may have eliminated sporozoites or infected hepatocytes[13]. Indeed an

80% reduction of liver-stage parasite burden following sporozoite challenge was shown in mice immunized with AMA-1[10]. After vaccination of humans with AMA-1 and MSP-1, time to diagnosis, which was delayed in the vaccinees, significantly correlated with liver-to-blood parasite levels but not blood-stage multiplication rates. This suggests again that this vaccine may induce pre-erythrocytic rather than the originally intended blood-stage immunity[12]. These examples indicate that a vaccine formulation based on only one or two malarial antigens could induce cross-stage protective immunity. Whole organism vaccines that allow exposure to many parasite antigens should therefore provide greater potential for cross-stage immunity, which would enhance protection induced by pre-erythrocytic and blood-stage parasites. We therefore discuss evidence for cross-stage immunity from different whole parasite vaccination approaches, which offer the opportunity to identify as yet unknown cross-protective antigens for multi-stage malaria vaccine development.

Evidence for cross-stage immunity from pre-erythrocytic whole parasite vaccination approaches

The proteome of liver-stage parasites becomes increasingly similar to blood-stage parasites as liver development proceeds[9]. Furthermore the amount of parasite antigen increases as the parasite matures in hepatocytes. Killed sporozoites that fail to invade hepatocytes are incapable of inducing protection[18,19], suggesting that liver-stage development is indispensable for induction of protective immunity. Irradiation of sporozoites, which arrests their development early during the liver-stage[20,21], induces immunity to pre-erythrocytic stages only[18,22]. A very limited number of studies have, however, investigated whether there are significant immune responses, or any level of protection against blood-stage parasites. One report from Krzych *et al*.[23] suggests a more in-depth study of cross-stage immune responses induced by irradiation attenuated sporozoites might be valuable, as T cells from human volunteers immunized with irradiated sporozoites responded to both pre-erythrocytic and blood-stage antigens, including MSP-1. This response was greater in immunized volunteers, who were protected from challenge infection, than in unprotected volunteers and comparable to malaria-experienced individuals[23]. Therefore, immunization of humans with irradiated sporozoites leads to the induction of immune responses recognizing blood-stage antigens. CD8 T cells, which were shown to be essential for pre-erythrocytic protection following irradiated sporozoites immunization[24], proliferate more strongly in mice if in addition to *P. berghei* irradiated sporozoites they were exposed to parasitized erythrocytes[25].

This suggests that blood-stage infection can enhance pre-erythrocytic vaccine efficacy. Furthermore in a *P. berghei* infection model in mice, multiple booster immunizations with high numbers of irradiated sporozoites resulted in delayed patency and reduced peak blood-stage parasitemia after sporozoite challenge (Nganou-Makamdop K, personal communication), suggesting that cross-stage protective responses targeting blood-stage parasites may have developed.

Targeted deletion of parasite genes important for liver-stage development is an alternative strategy to arrest parasite development in hepatocytes. Similar to irradiated and chemically attenuated[26] sporozoites, immunization with knock-out parasites that arrest during the early liver-stage (e.g. *uis* 3[27], *uis* 4[28], *p36p*[29], *sap-1*[30], *p52*/*p36*[31,32]) results in pre-erythrocytic immunity. Late arrest during liver-stage development[33], however, appears to increase the chance of cross-stage immunity: Immunization with *P. yoelii fabb/f* knockout sporozoites can control and clear blood-stage parasitemia following challenge with parasitized erythrocytes[34]. This is the first direct evidence that immunization with an attenuated parasite, which does not develop beyond liver-stage, can elicit blood-stage immunity.

Cross-stage immunity therefore appears to be more efficient if liver-stage parasites arrest late in development as their antigenic profile becomes similar to blood-stage parasites[9] and the amount of antigen increases (**Figure 1**). Hence antigens expressed in late liver-stage parasites are, under certain conditions, capable of mediating not only pre-erythrocytic protection, but also reduce the risk of blood-stage breakthrough infection by inducing effective blood-stage immunity.

Evidence for cross-stage immunity from whole blood-stage parasite vaccination approaches

Plasmodium replicates massively in the liver such that one infected hepatocyte can release up to up to 30,000 blood-stage parasites[35]. Also due to their subsequent exponential multiplication blood-stage parasites are hence much more numerous than pre-erythrocytic parasites, which increases their potential to present protective antigens successfully. The possibility that immune responses against these antigens might not be only specific for blood-stage parasites, but could also target pre-erythrocytic stages has however hardly been investigated (**Figure 1**).

Disruption of the *purine nucleoside phoshphorylase* gene (*pnp*)[36] or *nucleoside transporter 1* (*nt1*) gene[37] in *P. yoelii* gives rise to severely attenuated blood-stage infections, and mice that had undergone an infection with these knockout parasites did not develop detectable patent parasitemia after infectious mosquito bite or sporozoite challenge[36,37]. This could represent effective blood-stage or pre-

erythrocytic immunity, since a reduction in liver parasite burden, which is the only direct evidence for pre-erythrocytic protection, was not shown. Direct evidence for pre-erythrocytic protection elicited by blood-stage parasites comes from mice that received a prophylactic treatment with chloroquine and were simultaneously infected with *P. yoelii* blood-stage parasites. Liver parasite burden is significantly reduced in these mice following sporozoite challenge[38].

It is presumed that the main purpose of blood-stage components in a multi-stage vaccine is to protect against breakthrough blood-stage infection[39], if the pre-erythrocytic components are only partially effective. However, if whole parasite blood-stage immunizations have the potential to also induce cross-stage protective responses enhancing immunity against pre-erythrocytic stages, they would be even more valuable for multi-stage malaria vaccine development.

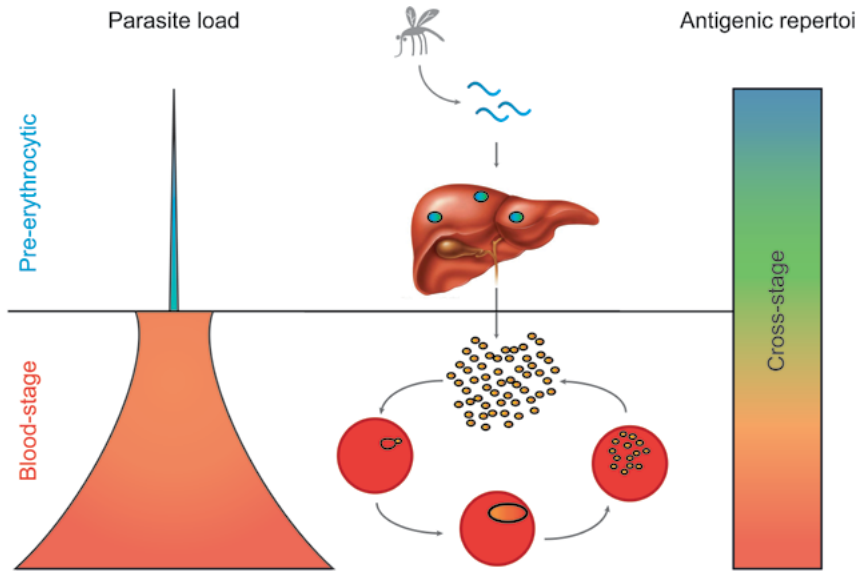

Figure 1 | Shared antigens can mediate cross-stage immunity after whole parasite vaccination
Malaria infection is initiated by an infectious mosquito bite, which inoculates a few sporozoites into the skin. Sporozoites then migrate to the liver and invade hepatocytes. During pre-erythrocytic development parasite load slowly increases as *Plasmodium* matures in the liver. In addition, the antigenic repertoire becomes increasingly similar to blood-stage parasites[9] and cross-stage antigens are expressed. Since up to 30,000 merozoites can be released from a single infected hepatocyte[1] parasite load rises rapidly when blood-stage parasites are released from the liver. Blood-stage parasites mature from rings to trophozoites and schizonts and parasite load increases exponentially with each new replication cycle. Apart from typical blood-stage antigens infected erythrocytes also express cross-stage antigens, which are shared with pre-erythrocytic parasites. There is evidence from whole parasite immunization approaches that shared antigens between pre-erythrocytic and blood-stage parasites could induce cross-stage immunity. Characterization of these antigens would greatly facilitate multi-stage malaria vaccine development.

Is cross-stage immunity responsible for the unprecedented efficiency of chemoprophylaxis with sporozoites immunization?

The induction of cross-stage immunity should be facilitated by exposure to both pre-erythrocytic and blood-stage parasites during immunization. Chemoprophylaxis with sporozoites (CPS) immunization, which uses infectious wild-type sporozoites combined with prophylactic antimalarial drug treatment (often chloroquine), allows the immune system to experience all vertebrate *Plasmodium* life-cycle stages including sporozoites, infected hepatocytes and parasitized erythrocytes. CPS immunization was first described in rodents[40,41] and was later shown to induce long lasting sterile protection against homologous mosquito-bite challenge in human volunteers[42-44]. In humans CPS immunization is about 20 times more efficient than immunization with irradiated sporozoites[22,45]. We therefore hypothesize that the completion of liver-stage development and the exposure to blood-stage parasites[38] during CPS immunization may enhance the protective efficacy by inducing protective cross-stage responses. CPS using primaquine, which primarily targets liver-stage parasites, substantially reduces the number of sterilely protected mice thereby strengthening this hypothesis[19]. In further support of this, mice that have experienced a self-cured infection with *P. chabaudi* blood-stage parasites derived from a donor mouse infected by mosquito bite have a substantially reduced liver-parasite burden following mosquito bite challenge[46]. Cross-stage immunity elicited by blood-stage parasites may hence contribute to the unprecedented efficiency of CPS immunization to induce sterile pre-erythrocytic protection in human volunteers[42-44]. Importantly exposure to blood-stage parasites during CPS immunization may significantly contribute to the observed pre-erythrocytic protection against mosquito bite challenge, but does not appear to protect against direct blood-challenge within the first four cycles of blood-stage replication[43].

Conclusion: Importance of studying cross-stage immunity for malaria vaccine development

Cross-stage immunity would be a powerful means to improve protective efficacy of malaria vaccines, however data to support this hypothesis are sparse. During liver-stage development both the amount of antigen and the antigenic similarity to erythrocytic parasites increases (**Figure 1**). Thus as *Plasmodium* matures in the liver, it is potentially capable of inducing immunity not only against pre-erythrocytic but also against blood-stage parasites[34]. Equally, exposure to attenuated blood-stage parasites could protect against sporozoite challenge[38].

Future studies using whole parasites, or indeed any other vaccination approaches should therefore investigate the potential of cross-stage immunity directly. Pre-erythrocytic vaccines should evaluate blood-stage protection after direct injection of parasitized erythrocytes, while blood-stage vaccines should demonstrate whether liver-parasite burden is reduced following sporozoite challenge. If cross-stage immune responses can be elicited, target antigens and crucial immunological mechanisms mediating it should be characterised using proteome-wide screening approaches (immunomics) of antibody and T cell reactivity comparing protected and unprotected individuals[47,48]. Selecting antigens capable of inducing cross-stage protection could greatly facilitate the development of a multi-stage malaria vaccine by increasing potency.

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CHAPTER 3

Blood-stage immunity to *Plasmodium chabaudi* malaria following chemoprophylaxis and sporozoite immunization

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Abstract

Protection against malaria in humans can be achieved by repeated exposure to infected mosquito bites during prophylactic chloroquine treatment (chemoprophylaxis and sporozoites (CPS)). We established a new mouse model of CPS immunization to investigate the stage- and strain-specificity of malaria immunity. Immunization with *Plasmodium chabaudi* by mosquito bite under chloroquine cover does not generate pre-erythrocytic immunity, which is acquired only after immunization with high sporozoite doses. Instead, CPS immunization by bite elicits long-lived protection against blood-stage parasites. Blood-stage immunity is effective against a virulent, genetically distinct strain of *P. chabaudi*. Importantly, if exposure to blood-stage parasitemia is extended, blood-stage parasites induce cross-stage immunity targeting pre-erythrocytic stages. We therefore show that CPS immunization can induce robust, long-lived heterologous blood-stage immunity, in addition to protection against pre-erythrocytic parasites following high dose sporozoite immunization. Cross-stage immunity elicited by blood-stage parasites may further enhance efficacy of this immunization regimen.

Introduction

Protective immunity against microorganisms is developed after repeated infection and recovery[1]. Vaccines are usually successful if they mimic these naturally acquired immune responses[2,3]. While protection against viruses and bacteria can be induced by vaccination with killed (inactivated) or live-attenuated pathogens[4], there is no licensed vaccine for human parasitic diseases like malaria that pose a major global health burden.

The apicomplexan malaria parasite *Plasmodium* is transmitted by bites of female anopheline mosquitoes. In the vertebrate host, sporozoites injected into the dermis migrate to the liver, where they establish a clinically silent infection of hepatocytes. Merozoites are then released from the liver and invade erythrocytes, leading to an exponential asexual replication cycle that is entirely responsible for the clinical signs and symptoms associated with malaria. Immunity against severe disease can be acquired following repeated infection, but sterile parasite clearance is rarely achieved[5]. Clinically immune adults in endemic areas still harbor parasites in their blood-stream[6]. These asymptomatic carriers also develop gametocytes, the form transmissible to mosquitoes, thereby allowing the parasite to complete its life-cycle.

To achieve malaria control and eventually eradication, transmission must be blocked[7]. A vaccine that protects against pre-erythrocytic parasites, and thus outperforms naturally acquired immunity, would greatly facilitate this aim. Parasites that arrest during the liver-stage, either because of irradiation[8] or targeted gene deletion[9], can provide immunity against challenge infection. Indeed, immunization of human volunteers with irradiated sporozoites can induce sterile protection in experimental settings[10,11]. However, in the absence of acquired immunity to the blood-stage parasite a pre-erythrocytic vaccine that is only partially effective, and therefore permits breakthrough erythrocytic infections, will provide no protection against severe malaria[12]. The inclusion of a blood-stage component together with an effective pre-erythrocytic vaccine is therefore preferred to provide a multi-stage malaria vaccine that minimizes both transmission and disease[13,14].

A recently described experimental malaria immunization protocol using chemoprophylaxis and sporozoites (CPS)[15] ensures exposure to pre-erythrocytic and blood-stage parasites, and hence has the unique potential to induce protection against all *Plasmodium* life-cycle stages in the vertebrate host. Three immunizations with bites of 10-15 *P. falciparum*-infected mosquitoes under chloroquine chemoprophylaxis are sufficient to elicit sterile protection against homologous challenge in human volunteers[15-17]. Although it is not possible

to measure liver parasite burden in human volunteers directly, it appears that immunity exclusively targets pre-erythrocytic parasite life-cycle stages, as there is no protection against direct blood challenge[17]. CPS immunization is thus substantially more effective than immunization with irradiated sporozoites, which requires 1,000 mosquito bites[10] or 5 *intravenous* (*iv*) injections of more than 100,000 sporozoites for sterile protection[11]. We therefore hypothesize that transient blood-stage parasitemia, before abrogation by chloroquine, may contribute to immunity following CPS immunization.

Here we have investigated the stage- and strain-specificity of protection in a novel mouse model of CPS immunization using *Plasmodium chabaudi*. *P. chabaudi* establishes a chronic, non-lethal blood-stage infection, which has been used extensively to characterise the immune response to blood-stage parasites *in vivo*[18]. A recently optimized protocol for *P. chabaudi* mosquito transmission[19] allows us now to also study pre-erythrocytic stages of this rodent parasite. Heterologous protection can readily be assessed since many genetically distinct *P. chabaudi* isolates displaying a variety of virulence phenotypes are available[20,21]. We immunized C57BL/6 mice three times with bites of *P. chabaudi* infected mosquitoes under oral chloroquine chemoprophylaxis similar to human clinical trials[15-17]. This approach is unique amongst all published animal models of CPS immunization[22-31], which have (without exception) used *iv* injection of high numbers of *P. berghei* or *P. yoelii* sporozoites for immunization. Furthermore rather than evaluating effector mechanisms by challenging shortly after immunization[22,26,27], we performed the challenge 100 days after the final immunization to test the generation and maintenance of long-term immunological memory.

CPS immunization with *P. chabaudi* by mosquito bite does not generate pre-erythrocytic immunity, which is acquired only after immunization with high doses of sporozoites. Instead, immunization by bite elicits blood-stage immunity that is effective against the immunizing strain and also a more virulent, genetically distinct *P. chabaudi*. Moreover, extended exposure to blood-stage parasitemia elicits robust pre-erythrocytic immunity, comparable to protection afforded by high dose sporozoite immunization. Exposure to blood-stage parasites thus elicits heterologous blood-stage immunity and can contribute to the pre-erythrocytic efficacy of this immunization regimen. Therefore these findings add significantly to advances from previous CPS immunization mouse models by evaluating the generation of immune memory after immunization with *P. chabaudi* by mosquito bite[22-31]. This is relevant for our understanding of acquired immunity in a malaria endemic setting, and can inform multi-stage malaria vaccine development.

Materials and Methods

Mice

Inbred C57BL/6 mice, originally obtained from Jackson Laboratories (Bar Harbor, USA), were bred under specific pathogen-free conditions at the MRC National Institute for Medical Research (NIMR) for over 30 years. All experiments were performed in accordance with UK Home Office regulations (PPL 80/2358) and approved by the ethical review panel at the MRC NIMR. Mice were housed under reverse light conditions (light 19.00-07.00, dark 07.00-19.00 GMT) at 20-22°C and 50% relative humidity, with continuous access to mouse breeder diet and water.

Parasites and Mosquitoes

Plasmodium chabaudi chabaudi (*P. chabaudi*) AS and CB were cloned at the University of Edinburgh and sent to the NIMR in 1978 and 1982 respectively. Both parasite lines were routinely serially blood-passaged through mice between 26-32 times by intraperitoneal (*ip)* injection of parasitized erythrocytes or mosquito transmitted according to a recently published protocol[19]. In brief C57BL/6 mice were injected *ip* with 100,000 parasitized erythrocytes and 14 days post infection gametocytemia was assessed on Giemsa-stained (VWR, Lutterworth, UK) thin blood film. *A. stephensi* mosquitoes, pre-treated with 50µg/ml gentamicin (Sigma, Gillingham, UK) and starved for 24h before transmission, were fed on anaesthetised mice with >0.1% gametocytes of total erythrocytes at a ratio of >1 mouse per 100 mosquitoes. Mosquitoes were kept at 26.0°C (+/− 0.5°C) in an ultrasonic humidity cabinet and provided with 8% Fructose and 0.05% 4-Aminobenzoic acid (both Sigma, Gillingham, UK) feeding solution. After 8 days a sample of 20 mosquitoes were dissected to assess development of *P. chabaudi* oocyts in the midgut. For infection of experimental mice 20-23 mosquitoes were transferred into 25cl paper cups after 14 days, starved for 24h and fed on anaesthetized mice for 20-25min at room temperature. Typically mice were exposed to 9.15 (Median, Range 6.9-13.6) *P. chabaudi* infected mosquito bites[19].

Isolation of sporozoites

Sporozoites were isolated from *P. chabaudi* infected mosquito salivary glands 15 or 16 days post gametocyte feed. Salivary glands were dissected under a stereomicroscope, transferred to a glass homogenizer and kept in RPMI supplemented with 0.2% Glucose, 0.2% Sodium bicarbonate (both Sigma, Gillingham, UK), 2mM L-Glutamine (Gibco, Paisley, UK) and 10% fetal bovine serum

(GE Healthcare Life Sciences, Pittsburgh, Pennsylvania), on ice for maximum 2h. Sporozoites were released from the glands by gentle homogenization and washed 3 times before enumeration. The number of sporozoites per infected mosquito was enumerated for each mosquito transmission experiment. For *iv* injection *P. chabaudi* CB sporozoites were used, since mosquitoes infected with this parasite strain harbor an increased number of sporozoites per infected mosquito in their salivary glands (Median 1638, Range 175-2576) compared to *P. chabaudi* AS (Median 438, Range 43-956)[19].

Attenuation of sporozoites by irradiation

To arrest parasite development in the early stages of liver development *P. chabaudi* CB infected *A. stephensi* were exposed to 16 Gray (=16,000 rad)[25] of Caesium-137 γ-irradiation 15 or 16 days post gametocyte feed just prior to sporozoite dissection.

Chemoprophylaxis and sporozoite (CPS) immunization and challenge regimen

Female age-matched 8-10 week old C57BL/6 mice were infected three times in two-week intervals with *P. chabaudi*: either by *P. chabaudi* AS infected mosquito bites or *iv* injection of *P. chabaudi* CB sporozoites. Mice were treated after each immunization with 100mg/kg chloroquine diphosphate salt (chloroquine, Sigma, Gillingham, UK) by gavage daily for 10 days, starting from the day of mosquito transmission. Mock immunized mice received uninfected mosquito bites and chloroquine treatment. 100 days after the last CPS immunization mice were challenged with *P. chabaudi* AS or *P. chabaudi* CB infected mosquito bites, or via *ip* injection of 100,000 parasitized erythrocytes (direct blood-challenge) that were obtained from either a donor mouse infected by mosquito bite or after serial blood passage. Since each erythrocytic cycle of *P. chabaudi* is approximately 24h long[18] development of blood-stage parasitemia was monitored daily by microscopy of Giemsa-stained thin blood films, from erythrocytic cycle 3 to 14 and every other day thereafter. The limit of detection was 0.01% parasitemia, which equals 1 parasitized red blood cell in 10,000 erythrocytes or 1,000,000 parasitized erythrocytes per ml of blood.

Quantification of liver- and blood-stage parasitemia by quantitative Real Time PCR

Liver and blood-parasitemia was assessed by quantifying 18S rRNA using qRT PCR. 42h after mosquito bite challenge mice were terminally anaesthetized

and immediately upon cessation of respiration their livers were perfused with 5ml RNAse-free Phosphate buffered saline (PBS, Gibco, Paisley, UK). Using the Gentle MACS homogenizer (Miltenyi, Bisley, UK) the whole liver was homogenized in 4ml Guanidinium thiocyanate (Sigma, Gillingham, UK) solution[32] and 600µl aliquots were stored at -80°C. To assess blood-parasite burden during CPS immunization and in the first erythrocytic cycles following bite challenge 10µl of blood were isolated from the tip of the mouse-tail and after 2 washes in RNAse-free PBS stored at -80°C in 100µl Guanidinium thiocyanate solution[32]. The first sample was taken either just before (erythrocytic cycle 0) or 20h after liver merozoite egress (erythrocytic cycle 1) and then every 24h for 4 days. Since *P. chabaudi* displays a synchronous infection[18] all blood-stage parasites analyzed were therefore at the late trophozoite stage of development. RNA was extracted from liver- as well as blood-samples using the Guanidinium-thiocyanate-phenol-chlorophorm method (all Sigma, Gillingham, UK)[32]. RNA was thereafter reverse transcribed by PCR (Temperature profile: 25°C for 10min, 42°C for 20min, 98°C for 5min) using 75U MuLV Reverse Transcriptase, 30U RNAse Inhibitor and 2.5µM Random Hexamer primers (all Applied Biosystems, Paisley, UK) per sample. The amount of 18S rRNA copies was quantified by Real-Time PCR using TaqMan Universal PCR Master Mix (Applied Biosystems, Paisley, UK), 300**η**M forward primer (5'-AAGCATTAAATAAAGCGAATACATCCTTAT-3'), 300**η**M reverse primer (5'- GGGAGTTTGGTTTTGACGTTTATGCG-3') and 50**η**M probe ([6FAM]CAATTGGTTTACCTTTTGCTCTTT[TAM]). All reactions were performed in the ABI 7900 HT Real Time PCR machine (Temperature profile: 50°C for 2min, 95°C for 10min, 40 cycles of 95°C for 15sec and 60°C for 1min). The amount of parasite 18S rRNA in the liver was calculated based on a Standard curve of known copy numbers of 18S rRNA. For every experiment liver-parasite burden was normalized to the mean burden of controls infected in the same experiment. Blood-stage parasitemia was quantified based on a Standard curve of 10-fold dilutions of mosquito transmitted *P. chabaudi* AS late trophozoites prepared identically to the samples.

Statistical analysis

Data were analyzed using GraphPad Prism v7. Unpaired data between two groups at a specific time point were analyzed by Mann-Whitney test (two-tailed, non-parametric). Differences between more than two groups were analyzed by non-parametric Kruskal-Wallis test with Dunn's multiple comparisons test. Significant differences are indicated by asterisks with *≤p0.05, **p≤0.01, ***p≤0.001.

Results

We investigated the stage- and strain-specificity of protection in a novel mouse model of CPS immunization **(Figure 1)**. C57BL/6 mice were immunized three times at 2-week intervals with *P. chabaudi* AS infected mosquito bites. For certain experimental questions it was necessary to deviate from the natural route of infection and inject sporozoites *iv* to control the dose. Starting on the day of infection, mice were then treated orally with 100 mg per kg chloroquine for 10 days following each immunization. To assess the long-term efficacy of acquired immunity, mice were challenged approximately 100 days after the last immunization. Protection against pre-erythrocytic or blood-stage parasites was evaluated after mosquito bite or direct blood challenge.

CPS immunization leads to a transient blood-stage infection

Transient blood-stage parasitemia is a key feature of CPS immunization. We used quantitative RealTime (qRT) PCR to measure erythrocytic parasite burden after each immunization with *P. chabaudi* AS infected mosquito bites under chloroquine cover. After the first immunization, approximately 50,000 parasites per ml whole

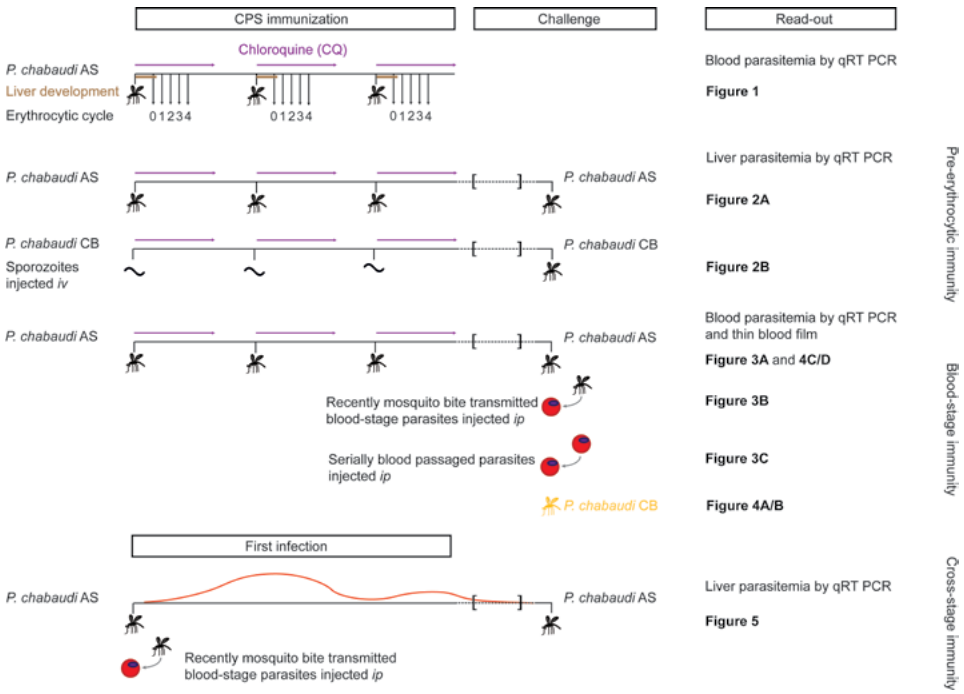

Figure 1 | Overview experimental procedures *(for legend see next page)*

Figure 1 | Overview experimental procedures *(see previous page)*
To quantify transient blood-stage exposure during chemoprophylaxis and sporozoite (CPS) immunization female C57BL/6 were immunized three times at 2-week intervals with *P. chabaudi* AS infected mosquito bites (typically 9.15 (Median, Range 6.9-13.6) [19]). Following each immunization mice received 100 mg per kg chloroquine (CQ) *per os* daily for 10 days, starting from the day of infection. A small blood sample was taken 48h after each mosquito transmission (before merozoite egress from the liver, *P. chabaudi* develops in the liver for 52h [18]; erythrocytic cycle 0), and then every 24h until erythrocytic replication cycle 4. Blood parasitemia was analyzed by sensitive quantitative RealTime (qRT) PCR (**Figure 2**).
Pre-erythrocytic immunity was evaluated in mice immunized three times with either *P. chabaudi* AS infected mosquito bites (**Figure 3A**) or by *intravenous* (*iv*) injection of defined numbers of *P. chabaudi* CB sporozoites (**Figure 3B**). *P. chabaudi* CB was used since mosquitoes infected with this parasite harbor an increased number of sporozoites in their salivary glands [19], which made injections of high numbers of sporozoites technically feasible. Mice were challenged 100 days after the last immunization by mosquitoes infected with the respective homologous strain. Liver parasitemia was examined 42h after challenge by qRT PCR.
Blood-stage immunity was assessed in mice immunized with *P. chabaudi* AS infected mosquito bites by qRT PCR and thin blood film following homologous challenge with either infected mosquito bites (**Figure 4A** and **5C/D**) or *intraperitoneal* (*ip*) injection of parasitized erythrocytes, which were either derived from a donor mouse infected by mosquito bite (recently mosquito transmitted, **Figure 4B**) or after 26-32 serial blood-passages (**Figure 4C**). Heterologous protection was assessed using *P. chabaudi* CB infected mosquito bites (**Figure 5A/B**).
To evaluate cross-stage protection mice received a first infection with *P. chabaudi* AS either by mosquito bite or by *ip* injection of recently mosquito transmitted parasitized erythrocytes. The resulting blood-stage infection was eventually self-cured without intervention. Mice were re-challenged 100 days after their first infection with *P. chabaudi* AS infected mosquito bites and liver parasitemia was evaluated by qRT PCR (**Figure 6**).

blood were detected within the first erythrocytic cycle (**Figure 2**). The amount of blood-stage parasites within the first erythrocytic cycle varied extensively (Median 47,596, Range 67 to 222,699), reflecting the stochastic inoculation of sporozoites during mosquito bite[33-35]. Thereafter, chloroquine reduced parasitemia by 86-96% every 24h. After the 4th cycle the majority of erythrocytic parasites were cleared. Similarly, after the second and third immunization mice experienced a substantial number of circulating blood-stage parasites for 48-72 hours. However, although blood-stage parasites were detected in all but one mouse, parasitemia in the first erythrocytic cycle was reduced by 5- and 13-fold after the second and third immunizations, respectively, when compared to infection controls (**Figure 2**). Consequently, one CPS immunization is sufficient to reduce blood-stage parasite burden within the first erythrocytic cycle, which indicates either pre-erythrocytic or blood-stage immunity.

Pre-erythrocytic immunity requires high doses of sporozoites during CPS immunization

In order to assess directly whether pre-erythrocytic immunity was generated by this CPS immunization protocol, liver parasite burden was analysed after mosquito bite challenge. Surprisingly, there was no difference in the liver parasite burden

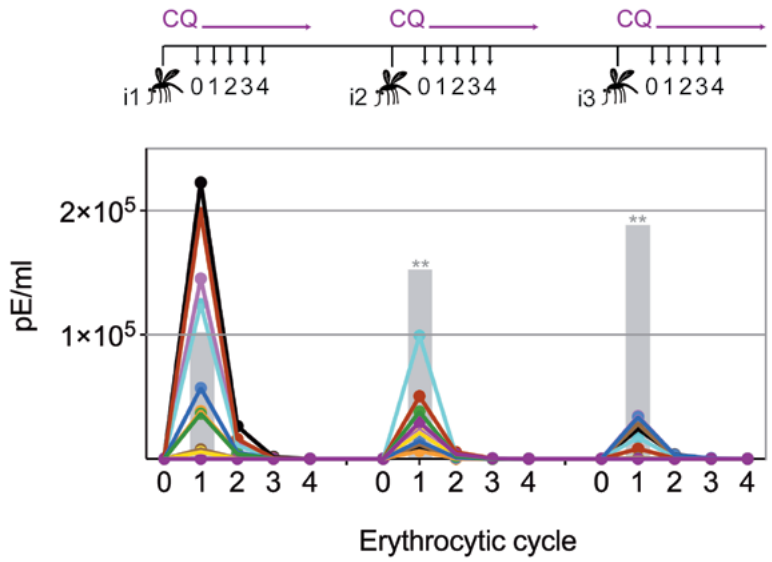

Figure 2 | Chloroquine permits transient blood-stage parasitemia during each immunization
The number of parasitized erythrocytes (pE) per ml of whole blood was enumerated by quantitative RealTime PCR after each CPS immunization (i1, i2, i3) with *P. chabaudi* AS infected mosquito bites under chloroquine (CQ) cover. The number of pE (at the late trophozoite stage) was quantified immediately before merozoite egress from the liver, at 48h post mosquito transmission (erythrocytic cycle 0), and then every 24h until erythrocytic replication cycle 4. Daily parasitemia of ten CPS immunized mice (each color represents an individual mouse) are shown. Blood-stage parasites were detected within the first erythrocytic cycle after every immunization in all but one mouse after the final immunization. Grey bars represent the mean parasitemia in the first erythrocytic cycle of naïve mice infected as controls for mosquito transmission efficiency separate with each immunization (n=3-5). Significant differences in the number of blood-stage parasites in the first erythrocytic cycle between naïve and CPS immunized mice are indicated (Mann Whitney test, ** p≤0.01).

between mice given three CPS immunizations with *P. chabaudi* AS infected mosquito bites and infection controls (**Figure 3A**). Therefore, immunization by mosquito bite under chloroquine cover according to this protocol failed to elicit pre-erythrocytic immunity. This is in contrast to results from other animal models of CPS immunization where pre-erythrocytic immunity is induced after high numbers of sporozoites are injected *iv*[25-29,31]. To test if pre-erythrocytic immunity can be induced after CPS immunization with large numbers of sporozoites we used *P. chabaudi* CB, since mosquitoes infected with this parasite strain harbor an increased number of sporozoites in their salivary glands compared to *P. chabaudi* AS[19], making these experiments technically feasible. In agreement with previous studies[25-29,31], mice immunized *iv* three times with 10,000 *P. chabaudi* CB sporozoites under chloroquine cover did show reduced liver parasite burden (up

to 90%) after mosquito bite challenge, compared to infection controls (**Figure 3B**). Conversely, mice immunized *iv* three times with a low dose of 100 *P. chabaudi* CB sporozoites (representative of the estimated number of *P. chabaudi* sporozoites that initiate infection via mosquito bite[19]) do not acquire pre-erythrocytic immunity (**Figure 3B**). It also appears that CPS immunization with 10,000 live sporozoites was more effective at inducing pre-erythrocytic immunity than immunization with 10,000 irradiated *P. chabaudi* CB sporozoites, which arrest during hepatic development[36] and do not establish a blood-stage infection (**Figure 3B**). This suggests that complete liver-stage maturation and the increased blood-stage parasitemia that accompanies immunization with 10,000 sporozoites (as compared to 100 sporozoites or mosquito bite) could contribute to pre-erythrocytic protection.

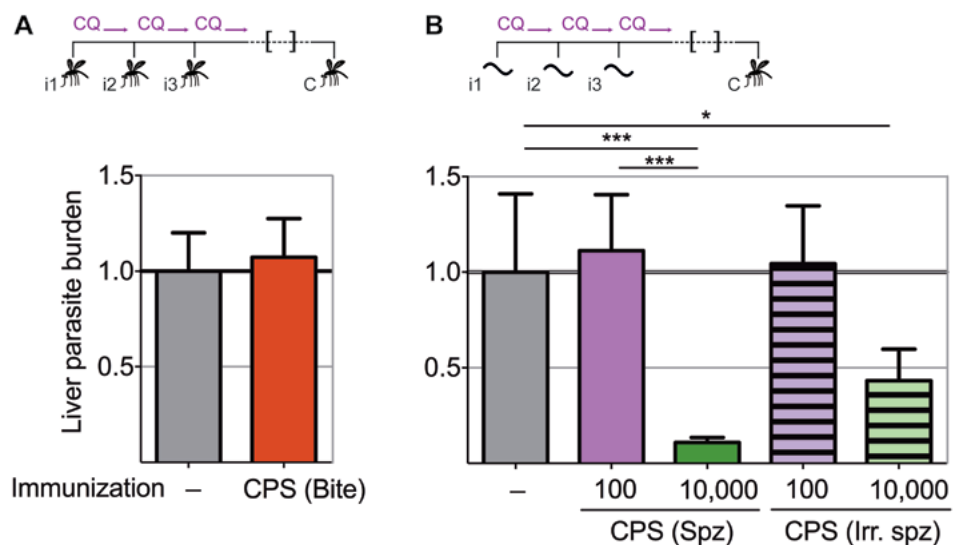

Figure 3 | Pre-erythrocytic immunity following CPS immunization requires high doses of sporozoites
Liver parasite burden was determined 42h after mosquito bite challenge as copy number of *P. chabaudi*-specific 18S rRNA. **A)** Mice were CPS immunized three times (i1, i2, i3) with *P. chabaudi* AS infected mosquito bites under chloroquine (CQ) cover (CPS (Bite)) and challenged (C) 96-104 days after immunization by bites of *P. chabaudi* AS infected mosquitoes (Pooled data from 3 independent experiments; naïve infection controls (-) n=25, CPS (Bite) n=35). **B)** 100 or 10,000 untreated or irradiated (Irr.) *P. chabaudi* CB sporozoites (spz) were injected *iv* three times under CQ cover. Mice were challenged 96 days after immunization by bites of *P. chabaudi* CB infected mosquitoes (naïve infection controls (-) n=12, all other groups n=20). All data are displayed relative to the mean of corresponding liver parasite burden of naïve infection controls and presented as Mean ± SEM, **A**) Mann-Whitney test: no significant difference between the groups; **B**) Kruskal Wallis with Dunn's multiple comparisons test *p≤0.05, ***p≤0.001.

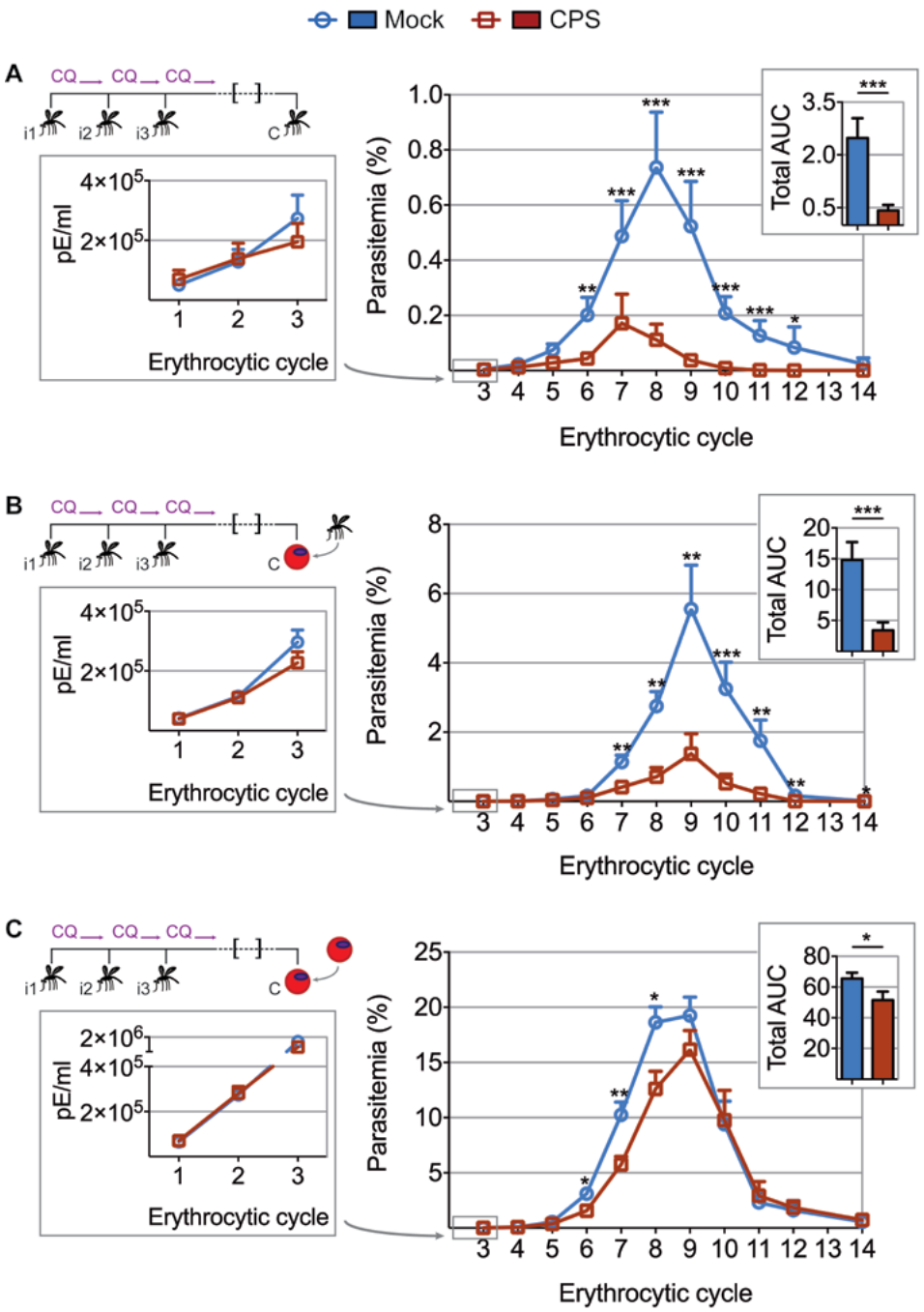

Figure 4 | CPS immunization elicits blood-stage immunity *(for legend see next page)*

Figure 4 | CPS immunization elicits blood-stage immunity *(see previous page)*
Mice were CPS immunized three times (i1, i2, i3) using chloroquine (CQ) and *P. chabaudi* AS infected or uninfected mosquito bites (mock immunized). Approximately 100 days after the final CPS immunization, mice were challenged (C) with *P. chabaudi* AS. Erythrocytic parasitemia was evaluated daily by quantitative RealTime PCR (cycle 1-3, displayed as parasitized erythrocytes (pE) per ml whole blood; **left**) and from cycle 3-14 by thin blood-film (expressed as % parasitized erythrocytes (parasitemia) 0.01% parasitemia corresponds to 1,000,000 pE per ml; **middle**). The total area under the curve (AUC) was calculated for each mouse between erythrocytic cycle 3 and 14 (**right**). **A)** Mosquito bite challenge: parasitemia from 1st to 3rd (n=10) and between 3rd and 14th erythrocytic cycle (representative of 3 independent experiments, n=12-19), total AUC between cycle 3 and 14 (n=19). **B)** Direct blood challenge using 10,000 erythrocytic parasites obtained from a donor mouse infected by mosquito bite; injected *ip*: parasitemia between 1st and 3rd (n=10) and 3rd and 14th erythrocytic cycle (representative of 3 independent experiments, n=10), total AUC between cycle 3 and 14 (n=10). **C)** Blood challenge using 10,000 serially blood passaged parasites; injected *ip*: parasitemia from 1st to 3rd (n=10) and between 3rd and 14th erythrocytic cycle (representative of 2 independent experiments, n=8-10), total AUC between cycle 3 and 14 (n=10). All data are presented as Mean ± SEM, Mann-Whitney test per time point *p≤0.05, **p≤0.01, ***p≤0.001.

THREE

CPS immunization by mosquito bite elicits blood-stage immunity

To test whether the transient blood-stage infection resulting from CPS immunization by mosquito bite (**Figure 2**) is sufficient to induce protection against erythrocytic parasites, we challenged mice approximately 100 days after the last immunization and measured blood-stage parasitemia (**Figure 4**). Mice that were CPS immunized with *P. chabaudi* AS had similar numbers of parasitized erythrocytes as compared to mock immunized controls within the first five erythrocytic cycles following mosquito bite challenge (**Figure 4A**). However, from erythrocytic cycle six parasitemia was significantly reduced and blood-stage parasites were cleared more rapidly in immunized mice. This was reflected in a 6-fold reduction of total area under the curve (AUC) (right inset, **Figure 4A**). Protection against erythrocytic parasites was also evaluated by direct blood challenge, using blood-stage parasites obtained from a donor mouse infected by mosquito bite. Similar to the results of mosquito bite challenge, blood-stage parasitemia was significantly reduced (**Figure 4B**), but the infection was still chronic in some mice (**Supplementary Figure 1**). However, blood-stage protection was abrogated when CPS immunized mice were challenged with serially blood passaged *P. chabaudi*; blood-stage parasites with increased virulence following multiple passages through naïve mice[37] (**Figure 4C**). In this case, CPS immunization reduced blood-stage parasite burden only between erythrocytic cycle 6 and 8, as compared to mock immunized controls. This was reflected in only a 1.2-fold reduction in total AUC (right inset, **Figure 4C**). Therefore, CPS immunization by mosquito bite elicits homologous blood-stage immunity, which is most effective in the context of mosquito transmission.

CPS immunization elicits heterologous blood-stage immunity

CPS immunization has so far not been shown to induce protection against challenge with genetically distinct strains of *Plasmodium*; a situation that would be encountered in human malaria-endemic areas. The genetic diversity amongst strains of *P. chabaudi*[20,21] allows us to investigate heterologous immunity in this model of CPS immunization. Mice that were CPS immunized with *P. chabaudi* AS infected mosquito bites had reduced peak parasitemia, and blood-stage parasites were cleared faster, when compared to mock immunized mice after homologous (*P. chabaudi* AS) and heterologous (*P. chabaudi* CB) mosquito-bite challenge (**Figure 5A-D**). A direct comparison between *P. chabaudi* AS and CB challenge revealed nonetheless that homologous blood-stage immunity is more effective

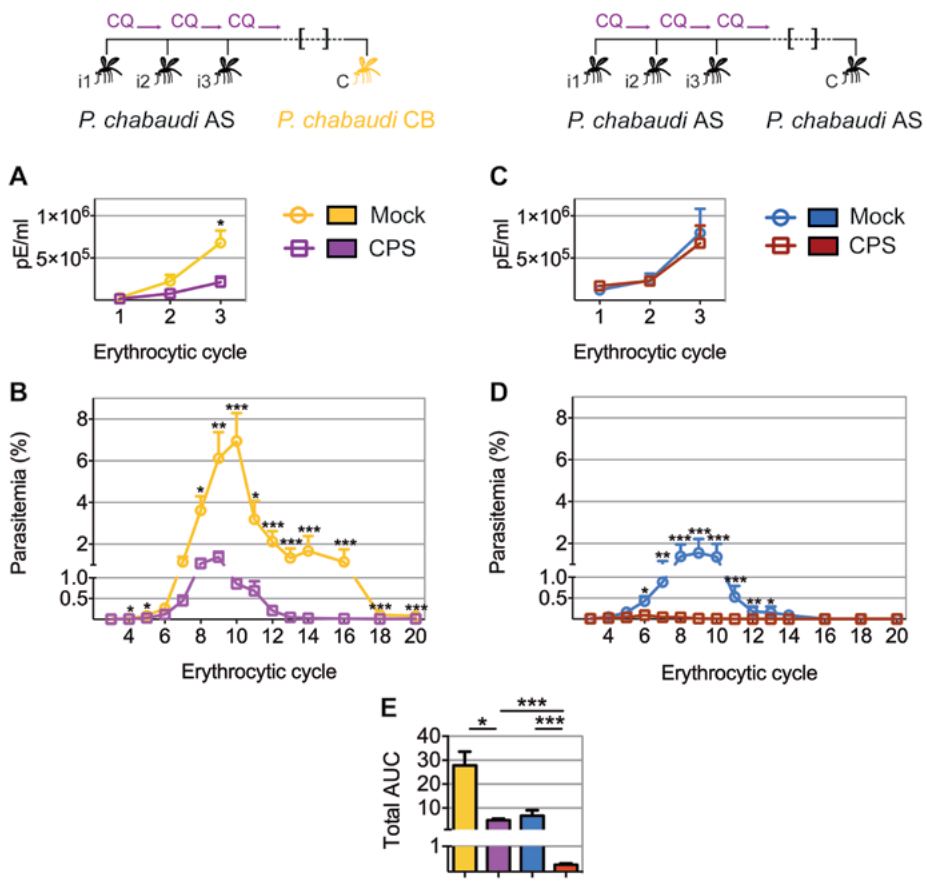

Figure 5 | CPS immunization elicits heterologous blood-stage immunity
(for legend see next page)

Figure 5 | CPS immunization elicits heterologous blood-stage immunity *(see previous page)*
Mice were CPS immunized three times (i1, i2, i3) under chloroquine (CQ) cover by *P. chabaudi* AS infected mosquito bites or mock immunized with uninfected mosquito bites, and challenged (C) 96 to 107 days later by mosquito bite. Erythrocytic parasitemia was evaluated daily by quantitative RealTime PCR for blood-stage parasites (cycle 1-3, displayed as parasitized erythrocytes (pE) per ml whole blood) and from cycle 3-20 by thin blood-film (expressed as % parasitized erythrocytes (parasitemia), 0.01% parasitemia corresponds to 1,000,000 pE per ml). **A/B** Heterologous challenge with *P. chabaudi* CB infected mosquitoes **A)** Parasitemia between 1st and 3rd (n=10) and **B)** from 3rd to 20th erythrocytic cycle (n=20). **C/D** Homologous challenge with *P. chabaudi* AS infected mosquitoes **C)** Parasitemia between 1st and 3rd (n=10) and **D)** from 3rd to 20th erythrocytic cycle (CPS immunized n=20, mock immunized n=19). **E)** Total AUC comparing mock and CPS immunized mice receiving heterologous or homologous mosquito bite challenge. Data are presented as Mean ± SEM, **A-D** Mann-Whitney test per time point **E)** Kruskal Wallis with Dunn's multiple comparisons test *p≤0.05, **p≤0.01, ***p≤0.001.

than heterologous immunity. In this experiment, CPS immunization reduced total AUC by 25-fold following homologous challenge, as compared to 6-fold following heterologous challenge (**Figure 5E**). Nevertheless, CPS immunization elicits blood-stage protection against a robust heterologous challenge with the genetically distinct, and more virulent, CB strain of *P. chabaudi*.

Extended exposure to blood-stage parasites elicits robust pre-erythrocytic immunity

Blood-stage parasites appear to be both the source and target of protection following CPS immunization with *P. chabaudi* AS infected mosquito bites. It was shown that immunization with serially blood passaged *P. yoelii* parasites and prophylactic chloroquine treatment can elicit pre-erythrocytic immunity[38]. We wanted to assess whether blood-stage parasites have the potential to induce pre-erythrocytic protection also in the context of mosquito transmission. We therefore asked whether a fulminant blood-stage infection could elicit cross-stage immunity against pre-erythrocytic parasites. We infected mice with *P. chabaudi* AS by mosquito bite or *ip* injection of recently mosquito transmitted blood-stage parasites. The two groups of mice were not drug-treated, and therefore experienced a low-grade chronic, recrudescing blood-stage infection for up to 90 days[37]. After mosquito bite challenge, both groups of mice demonstrated reduced liver parasite burden (up to 85%), compared to infection controls (**Figure 6**). Cross-stage immunity is therefore a powerful mechanism for protection against pre-erythrocytic parasites, which may be absent during CPS immunization with small sporozoite numbers as the blood-stage infection is curtailed by the use of chloroquine.

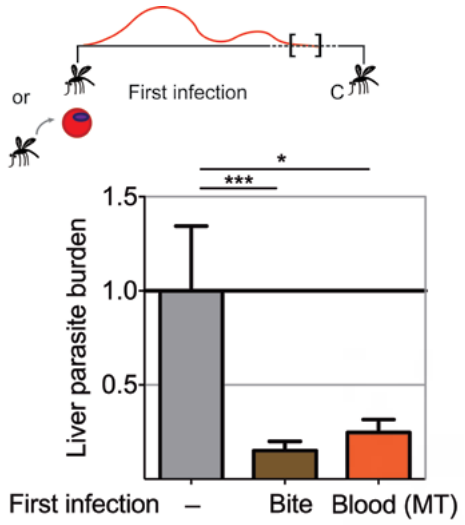

Figure 6 | Extended blood-stage parasitemia elicits pre-erythrocytic immunity
Mice received a first infection with *P. chabaudi* AS either by mosquito bite (Bite) or *ip* injection of 10,000 parasitized erythrocytes obtained from a donor mouse infected by mosquito bite (Blood (MT)). Blood-stage parasitemia was self-cured before challenge (C; 98 days post-infection) using *P. chabaudi* AS infected mosquito bites. Liver parasite burden was determined 42h after mosquito bite challenge as copy number of *P. chabaudi*-specific 18S rRNA. Data are displayed relative to the mean of corresponding liver parasite burden of naïve infection controls (-). Pooled data from 2 independent experiments (Naïve (-) and Bite n=30, Blood (MT) n=20) are presented as Mean ± SEM, Kruskal Wallis with Dunn's multiple comparisons test *p≤0.05, ***p≤0.001.

Discussion

Timing, route of infection and antigen dose play major roles in determining the initial priming of the antimalarial immune response[38-42]. We incorporated many of these aspects from human clinical trials[15-17] in a new *P. chabaudi* mouse model of CPS immunization to investigate the stage- and strain-specificity of CPS-induced protection against malaria. Our results highlight the complexity of immunity against the different life-cycle stages of the malaria parasite (**Table 1**). Pre-erythrocytic immunity appears to depend on the number of immunizing sporozoites. Here we find no evidence of pre-erythrocytic immunity after CPS immunization with *P. chabaudi* infected mosquito bites, which inoculate an estimated maximum of 100 sporozoites per immunization[19,37]. Sterile pre-erythrocytic protection was however reported in human CPS immunization trials[15-17]. *Anopheles stephensi* mosquitoes experimentally infected with *P. falciparum* 3D7 or NF54 habor 50-200 times more sporozoites in their salivary glands[17] compared to *P. chabaudi* AS[19]. However, only few sporozoites are injected into the skin during mosquito bite and this number is independent of salivary gland sporozoites load[33-35]. Since the number of sporozoites establishing a liver-stage infection can further be influenced by inherent sporozoite infectivity[43], our best estimate of immunizing sporozoite dose is the number of infected erythrocytes observed directly after egress from the liver. Assuming that 10,000 merozoites are released from one infected liver cell[44] we estimate approximately 400 infected hepatocytes (95% CI 137-1250) in human volunteers[17] compared to 5 in *P. chabaudi* AS immunized mice (95% CI 1-31). Therefore the number of infected hepatocytes after CPS immunization by mosquito bite in the *P. chabaudi* mouse model is approximately 100-fold lower than in CPS immunized humans. We show that this differences in infected hepatocyte numbers by a factor of 100 can be significant for the development of pre-erythrocytic immunity: Three immunizations with 10,000 *P. chabaudi* sporozoites *iv* induces long-lasting protection against mosquito bite challenge, while three immunizations with 100 *P. chabaudi* sporozoites fails to do so. This is in general agreement with other rodent malaria studies using *P. berghei*[22-26,31] or *P. yoelii*[27-30], in which sterile pre-erythrocytic immunity is observed after immunization with high sporozoite doses (typically 10,000-50,000 sporozoites per immunization), while a reduction in sporozoite dose or the number of immunizations leads to breakthrough blood-stage infections upon challenge[27,29]. Reduction of the number of *P. falciparum* infected mosquitoes also reduces the frequency of sterilely protected volunteers[16], indicating that the number of sporozoites establishing a liver-stage infection fails to surpass the protective threshold to elicit sterile pre-erythrocytic immunity.

This may also explain why in malaria-endemic areas pre-erythrocytic immunity is thought to be absent[45]. In addition to maximizing specific responses against immunodominant antigens, CPS immunization with high numbers of sporozoites may broaden the immune repertoire by including protective responses against subdominant antigens, which could enhance heterologous pre-erythrocytic protection[46]. This may further be enhanced by the longer liver-stage development of *P. falciparum* (egress 6.8 days after mosquito bite[47]) compared to *P. chabaudi* (egress after 52h[18]). Longer liver stage development may positively influence the generation of pre-erythrocytic immunity by allowing time for protective immune responses to develop.

A key feature of CPS immunization is that it permits exposure to all *Plasmodium* life-cycle stages in the vertebrate host, including parasitized erythrocytes. It is well known that repeated exposure to blood-stage parasites, for example in rodent models[39,40,48], in humans exposed to ultra-low doses of parasitized erythrocytes while receiving drug treatment[49], during malaria-therapy of neurosyphilis patients[50], and in people living in malaria-endemic areas[51], induces blood-stage immunity. Our results also clearly show that during CPS immunization repeated transient blood-stage infection (less than 0.01% parasitemia for 48-72 hours) elicits long-lasting blood-stage immunity. Protection against challenge infection was only apparent after multiple erythrocytic replication cycles and patent blood-stage parasite densities, which could indicate that blood-stage protection was not observed in CPS immunized human volunteers after direct blood-challenge because drug treatment is required at low parasite densities as soon as patency is reached (typically between the 3rd and 4th erythrocytic cycle[17]). Blood-stage parasites are however recognized in human volunteers, which was demonstrated by an earlier increase of IFNγ and monokines induced by IFNγ (MIG) concentrations in CPS

Table 1 | Relationship between the dose of immunizing pre-erythrocytic and blood-stage parasites and the acquisition of immunity

	Low dose	High dose
Sporozoites (Liver-stage parasites)	Mosquito bite or 100 sporozoites *iv*: → No pre-erythrocytic immunity **Figure 3A/B**	10,000 sporozoites *iv*: → Pre-erythrocytic immunity **Figure 3B**
Blood-stage parasites	Blood-stage infection curtailed by chloroquine: → Partial blood-stage immunity **Figure 4**	Fulminant, self-cured blood-stage infection: → Blood-stage immunity [37] → Pre-erythrocytic immunity **Figure 6**

immunized volunteers compared to infection controls after direct blood challenge[17]. It will be of value to investigate whether the observed blood-stage protection in this mouse model is also detected in CPS immunized primates, where a longer blood-stage infection than that allowed in human clinical trials is possible.

Despite the reduced peak parasitemia and faster clearance of blood-stage parasites during the acute phase of infection in CPS immunized mice, recrudescent parasitemia could still be observed in the chronic phase of infection after challenge, suggesting a parasite variant escapes the protective immune response[52]. There are very few reports on protective efficacy of CPS immunization (or indeed any sporozoite-based vaccine) against direct blood-challenge[27-29]. Doll *et al*.[30] reported that sustained, subpatent blood-stage infection after treatment with a commonly used dose of chloroquine can induce partial blood-stage protection. Low-grade transient blood-stage parasitemia, achieved by attenuation of blood-stage parasites using an antimalarial drug[40,49], a DNA alkylating agent[53] or genetic tools[54,55] similarly provides protection against homologous and heterologous blood-stage challenge.

We could show that CPS-induced blood-stage immunity is effective against heterologous mosquito bite challenge with a more virulent and genetically distinct strain of *P. chabaudi*[20,56]. In agreement with the observed cross-species protection after CPS immunization with mefloquine[29], and one study using chemically attenuated sporozoites for immunization[57], heterologous protection is less effective than homologous immunity. Nevertheless, CPS immunization can elicit long-lived protection against both homologous and heterologous blood-stage parasites, which will be important to minimize disease severity in the case of breakthrough blood-stage infections. This is essential for the development of an effective multi-stage malaria vaccine[12,14].

In stark contrast to the observed heterologous blood-stage protection after mosquito bite challenge infection, protection was almost completely abrogated following direct blood-challenge with virulent parasites obtained after continuous serial blood passage. This suggests that blood-stage parasites immediately after mosquito transmission express antigens not present on serially blood passaged parasites and that these antigens may be the target of protective immunity following CPS immunization. Serially blood passaged parasites can hence escape from CPS-induced blood-stage immunity. One group of *Plasmodium* genes, whose expression is altered by mosquito transmission during blood-stage infection is the *Plasmodium* interspersed repeat gene family (*pir*); termed *cir* in *P. chabaudi*[58]. Transcription of more than half of all *cir* genes is increased in blood-stage parasites

after mosquito transmission compared with their transcription after serial blood passage. This diversification of *cir* transcription is associated with a more effective host immune response, which in turn attenuates parasite virulence[37]. The *cir* genes could also be candidate targets for cross-stage immunity, as *P. berghei pir* genes are also transcribed during the liver stages (personal communication B.M. Franke-Fayard and C.J. Janse, Leiden University Medical Center, The Netherlands). An investigation into shared PIR proteins between liver and blood-stage parasites may hence provide valuable information for multi-stage malaria vaccine development. Furthermore absence of blood-stage protection in previous CPS models may have been due to challenge with serially blood passaged rather then recently mosquito transmitted blood-stage parasites. It is therefore always essential to evaluate blood-stage immunity in the context of mosquito transmission. As shared antigenic targets between liver- and blood-stage parasites have been described[59], the exciting possibility of cross-stage protection has been considered but only rarely assessed. Genetically modified *fabb/f*- sporozoites that arrest late in liver-stage development protect against *iv* challenge with 100 blood-stage parasites[60]. On the other hand, a blood-stage infection with serially blood passaged *P. yoelii*, drug-treated with chloroquine after 4-5 days, reduces liver parasite load upon *iv* challenge with 35,000 sporozoites[38]. In our model of CPS immunization by mosquito bite it is likely that repeated transient blood-stage infection during immunization elicits the observed blood-stage protection, although we cannot yet exclude that responses acquired against pre-erythrocytic antigens, which are shared with blood-stage parasites[59], contribute as well. Because of the low number of infected hepatocytes after CPS immunization with *P. chabaudi* infected mosquito bites this seems however unlikely. Nevertheless, we demonstrate unequivocally that extended exposure to blood-stage parasites is an effective stimulator of pre-erythrocytic immunity. Exposure to blood-stage parasites during CPS immunization may thus significantly contribute to the observed pre-erythrocytic protection in human volunteers[15-17]. Indeed, cross-stage immunity could be responsible for the unprecedented efficacy of CPS immunization compared to immunization with irradiated sporozoites[10,11], which arrest early during liver-stage development and never establish a blood-stage infection. While extending exposure to replicating blood-stage parasites by delayed drug administration is not possible in humans, the incorporation of chemically[53] or genetically[54,55] attenuated blood-stage parasites should be considered to further enhance the generation and maintenance of both pre-erythrocytic and blood-stage immunity. This makes CPS immunization a powerful tool for the development of an effective multi-stage malaria vaccine.

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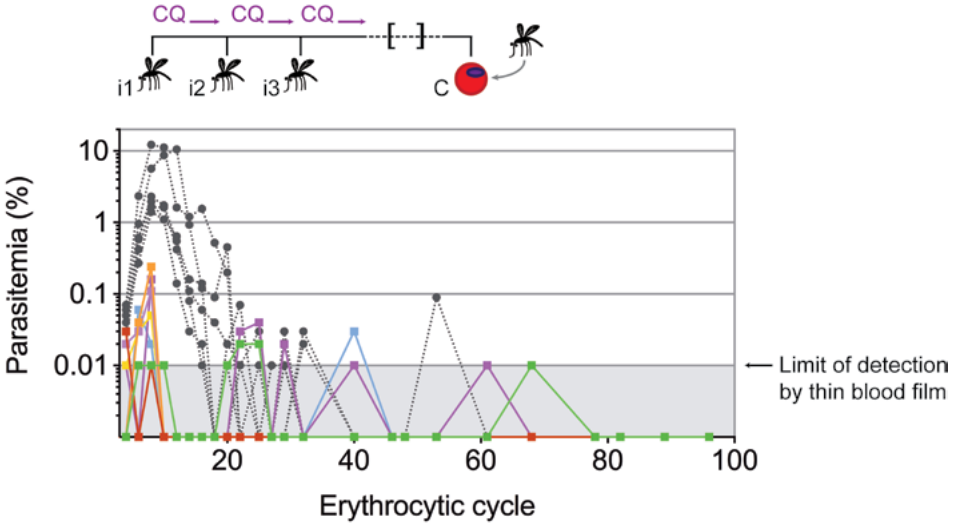
Supporting information



Supplementary Figure 1 | Chronic blood-stage infection in CPS immunized mice
Naïve infection controls or mice that were CPS immunized three times (i1, i2, i3) using chloroquine (CQ) and *P. chabaudi* AS infected mosquito bites were challenged (C) by *ip* injection of 100,000 parasitized erythrocytes derived from a mosquito bite initiated infection 100 days later. Parasitemia was evaluated by thin blood film for 96 erythrocytic cycles. Different colors represent individual CPS immunized mice (n=8), naïve infection controls are presented in grey with dashed lines (n=6). The limit of detection by thin blood film is 0.01% parasitemia (1 parasite in 10,000 erythrocytes or 1,000,000 parasites per ml blood).

CHAPTER 4

Memory B cell and antibody responses induced by *Plasmodium falciparum* sporozoite immunization

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Abstract

Background

Immunization of healthy volunteers under chemoprophylaxis with *Plasmodium falciparum* sporozoites (CPS immunization) induces sterile protection from malaria. Antibody responses are long known to contribute to naturally acquired immunity against malaria, but their association with sterile protection after whole sporozoite immunization is not well established. We therefore studied the induction and kinetics of malaria antigen-specific antibodies and memory B cells (MBC) during CPS immunization and their correlation with protection from challenge infection.

Methods

We assessed humoral reactivity to nine antigens representing different life-cycle stages of the malaria parasite by performing standardized MBC ELISpot and ELISA on peripheral blood mononuclear cells and plasma samples from 38 Dutch volunteers enrolled in two randomized controlled clinical trials (NCT01236612, NCT01218893).

Results

MBC and antibodies recognizing pre-erythrocytic and cross-stage antigens were gradually acquired during CPS immunization. The magnitude of these humoral responses did not correlate with protection, but directly reflected parasite exposure in CPS immunization and challenge.

Conclusions

Humoral responses to the malarial antigens circumsporozoite protein, liver-stage antigen-1, apical membrane antigen-1 and merozoite surface protein-1 do not to predict protection from challenge infection, but can be used as sensitive marker of recent parasite exposure.

Introduction

Malaria remains a major global-health burden leading to widespread morbidity and mortality, which is mainly caused by *Plasmodium falciparum*[1]. Apicomplexan *Plasmodium* parasites have a complex multi-stage life-cycle, initiated by anopheline mosquitoes depositing sporozoites into the skin of the vertebrate host, which then migrate to the liver where they establish a clinically silent infection of hepatocytes. After maturation, merozoites egress from hepatocytes into the blood stream where they invade and cyclically replicate within erythrocytes. During blood-stage infection, clinical pathology becomes apparent and can be severe. A safe, affordable and effective vaccine to supplement other intervention strategies would dramatically benefit public health[2], but remains elusive in spite of immense time and money investment[3], also due to our incomplete understanding of protective immunity[4].

Malaria subunit vaccine development has thus far yielded disappointing results; RTS,S being the only one ever tested in Phase III clinical trials. This circumsporozoite protein (CSP)-based vaccine showed an encouraging 50% sterile protection in malaria-naive adult volunteers[5], but only reduced clinical and severe disease by 30-45% in children in malaria endemic areas[6,7]. In contrast, using whole sporozoites as immunogens has the potential to 100% sterilely protect humans against malaria in experimental settings. These regimens often use irradiation-attenuated sporozoites, that cannot complete liver-stage development[8]. Twenty times more efficient than immunization with irradiated sporozoites, which requires bites by 1,000 mosquitoes[9] or at least 5 *intravenous* (*iv*) injections of 135,000 sporozoites for sterile protection[10], is immunization with fully infectious wild-type sporozoites delivered by mosquito bites combined with chloroquine chemoprophylaxis (Chemoprophylaxis and sporozoites, CPS). CPS immunization provides sterile and long-lasting protection [11,12] against pre-erythrocytic parasites (sporozoites and liver-stages)[13]. One potential reason for this unprecedented efficiency is the fact that in contrast to irradiation, chloroquine does not affect pre-erythrocytic parasite development[14], but only kills the pathogenic erythrocytic stage. CPS immunization is therefore an invaluable tool to systematically delineate mechanisms of protective immunity to malaria.

Antibodies play a critical role in preventing infection by a large range of pathogens[15]. Immediately after antigen encounter, antibodies are produced by short-lived plasma cells[16]. Long-term humoral immune memory, however, is only acquired if in addition both long-lived antibody-producing plasma cells and memory B cells (MBC) are generated[16-18]. MBC are activated upon antigen re-

encounter and rapidly develop into new antibody-producing cells that replenish the plasma cell pool[17]. In malaria, antibodies are mainly known for their ability to control erythrocytic parasites, thereby contributing to clinical immunity[19]. Their possible contribution to sterile, pre-erythrocytic immunity is less well established. In the present study, we therefore investigated the generation of malaria-specific MBC and antibody responses in CPS immunized volunteers, and assessed their association with sterile protection from challenge infection. We found that the magnitude of these responses, predominantly directed against pre-erythrocytic and cross-stage antigens, does not predict sterile protection from challenge infection, but is a sensitive read-out for the degree and nature of antigen exposure during immunization.

Materials and Methods

Human ethics statement

Both clinical trials, from which samples for this study were obtained, received approval by the Central Committee for Research Involving Human Subjects of The Netherlands (Study A, NL34273.091.10, Study B, NL33904.091.10) and were registered at ClinicalTrials.gov, number NCT01236612 (Study A) and NCT01218893 (Study B). The study team complied with the Declaration of Helsinki and Good Clinical Practice including monitoring of data. Volunteers enrolled in both studies provided written informed consent.

Clinical trial design

To determine the generation of malaria-specific MBC and antibody responses in CPS immunized and in primary infected individuals, we utilized peripheral blood mononuclear cell (PBMC) and plasma samples from two single-center randomized controlled clinical trials (**Figure 1**). In Study A[13], volunteers received bites from 15 *P. falciparum*-infected mosquitoes on three occasions, which was previously shown to sterilely protect from mosquito challenge[11,12], and were subjected to challenge with mosquito bites or parasitized erythrocytes injected *iv*. Study B[20] was a CPS immunization dose de-escalation study where volunteers were immunized thrice with bites from either 15 (3x15), ten (3x10) or five (3x5) *P. falciparum*-infected mosquitoes. All immunized and control subjects in this study were subjected to mosquito challenge. This study set-up allowed us to longitudinally assess humoral responses in healthy, previously malaria-naïve, adult volunteers before and after exposure to different *P. falciparum* life-cycle stages in the context of CPS immunization, in the presence of prophylactic drug levels, as well as after challenge infection. After challenge, sterile protection was defined as the absence of thick smear-detectable parasitized erythrocytes until 21 days post challenge. Volunteers who did show parasites by thick smear were immediately treated with antimalarial drugs, and all remaining volunteers received a presumptive drug-treatment 21 days after challenge. Importantly, the amount of exposure to blood-stage parasites during both immunization and challenge was characterized for each volunteer retrospectively by quantitative RealTime (qRT) PCR. For further details about study groups, outcomes and sample collection see supporting information.

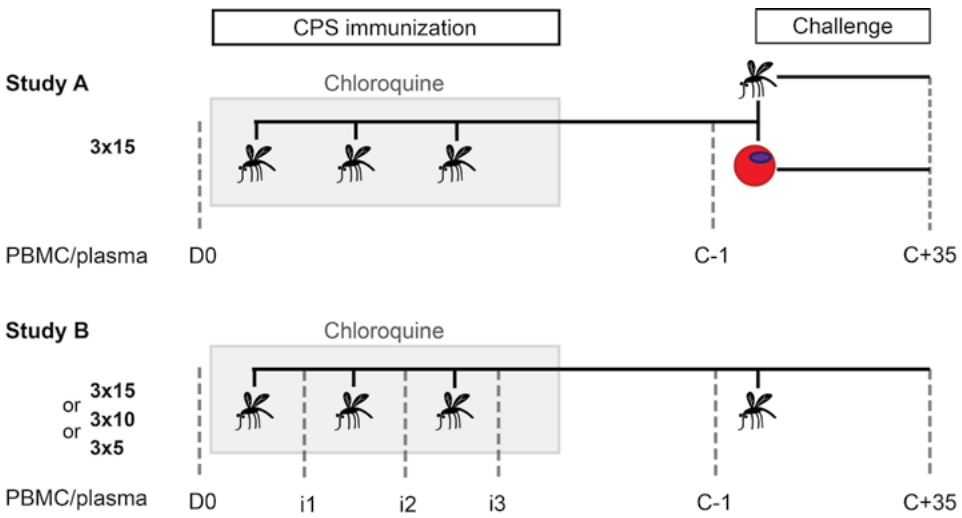

Figure 1 | Study design
Samples from two clinical chemoprophylaxis and sporozoites (CPS) immunization trials were analyzed. In **Study A**, volunteers were immunized three times with bites of 15 *Plasmodium falciparum*-infected mosquitoes every 4 weeks, while receiving a prophylactic regimen of the antimalarial drug chloroquine (grey box). For challenge infection 21 weeks after the last immunization (17 weeks after final chloroquine dose), immunized volunteers were split into two groups receiving either *P. falciparum*-parasitized erythrocytes or bites from 5 infective mosquitoes. Plasma and peripheral blood mononuclear cells (PBMC) were sampled prior to immunization (D0), one day before challenge (C-1) and 35 days after challenge (C+35). In **Study B**, three different immunization groups were exposed to bites from 3 times 15 (3x15), 10 (3x10) or 5 (3x5) *P. falciparum*-infected mosquitoes in 4-week intervals whilst receiving chloroquine prophylaxis. All groups were challenged 19 weeks after the last immunization (15 weeks after the last chloroquine dose) with 5 *P. falciparum*-infected mosquito bites. Plasma and PBMC samples were collected prior to immunization (D0), 28 days after the first (i1), second (i2) and third (i3) immunization, one day before challenge (C-1) and 35 days after challenge (C+35).

PBMC and plasma samples

PBMC were isolated by density centrifugation, cryopreserved at 10^7 cells/ml in fetal calf serum (FCS; Gibco)/10% DMSO (Merck) using Mr. Frosty freezing containers (Nalgene) and stored in vapor-phase nitrogen. Plasma samples were stored in aliquots at -20°C and re-thawed no more than twice.

Analysis of Memory B cell and antibody responses

The generation of malaria-specific MBC was assessed by MBC ELISpot assay[21]. Cryopreserved PBMC were thawed and stimulated for five days with the lectin Pokeweed Mitogen, *Staphylococcus aureus* Protein A, TLR9 ligand ODN 2006,

and recombinant human IL-10 to promote development of MBC into antibody secreting cells[22]. Antibody-secreting cells were then quantified by ELISpot analysis. Levels of malaria antigen-specific antibodies were determined in plasma as arbitrary units (AU) in relation to a pool of 100 sera from adults living in a highly endemic area in Tanzania by standardized enzyme-linked immunosorbent assay (ELISA)[11]. For a detailed description of both methods see supporting information.

Statistical Analysis

Data were analyzed using GraphPad Prism v6. Differences between two time points were analyzed by Wilcoxon matched-pairs signed rank test (two-tailed, non-parametric, paired). Unmatched data between two groups were analyzed by Mann-Whitney U test (two-tailed, non-parametric). Differences between more than two times points were analyzed by Friedman test with Dunn's multiple comparison post-hoc test (two-tailed, non-parametric, paired). Differences between two or more different groups over time were analyzed by repeated measures, mixed model two-way ANOVA with Bonferroni post-hoc test. Correlations were analyzed by Spearman correlation (non-parametric). Significant differences are indicated by asterices; * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), ns (not significant).

Results

Humoral responses against pre-erythrocytic and cross-stage malaria antigens are induced by CPS immunization

Malaria-specific antibody and MBC responses induced by the highest dose regimen of CPS immunization (bites from 3x15 infected mosquitoes, Study A and Study B, **Figure 1**) were analyzed for nine *P. falciparum* antigens representing either the pre-erythrocytic or the erythrocytic part of the *Plasmodium* life-cycle. circumsporozoite protein (CSP) and liver-stage antigen (LSA)-1 are exclusively expressed by sporozoites and liver-stage parasites, whereas most proteins including exported protein (EXP)-1, thrombospondin related anonymous protein (TRAP), the merozoite surface proteins (MSP)-1 and -2, apical membrane antigen (AMA)-1 and glutamate rich protein (GLURP), are most highly abundant in blood-stage parasites but are also expressed in late liver-stages (cross-stage antigens). Erythrocyte binding protein (EBA)-175 on the other hand is only expressed in blood-stage parasites. Nineteen to 21 weeks after the last CPS immunization (day before challenge: C-1, **Figure 1**) high antibody titers to the pre-erythrocytic antigens CSP and LSA-1, but no antibodies to the erythrocytic antigen EBA175 were detectable (**Figure 2A** and **C**). Antibodies for the cross-stage antigens EXP-1, MSP-1 and MSP-2 were induced, whereas no significant induction of AMA-1, GLURP and TRAP antibodies was observed (**Figure 2B**). All immunized volunteers developed CSP-specific antibodies (above group-background), and antibodies were detectable in 74% of volunteers for LSA-1. Over half (53%) of the volunteers had MSP-1-specific antibodies above background after CPS immunization, while 42% developed antibodies against EXP-1 and 37% against MSP-2. Immunization also gave rise to CSP-specific MBC in 95% of volunteers, and 42% of volunteers also developed MSP-1-specific MBC, while no MBC specific for any other selected antigen were observed (data not shown).

In the absence of parasite exposure antimalarial antibody responses contract, but memory B cells are maintained

We next studied the kinetics of acquisition of antibody and MBC responses to the strongest recognized antigens, CSP and MSP-1 over the course of CPS immunization (Study B, **Figure 1**). CSP- and MSP-1-specific antibody responses increased step-wise after the first, second and third immunization (**Figure 3A**).

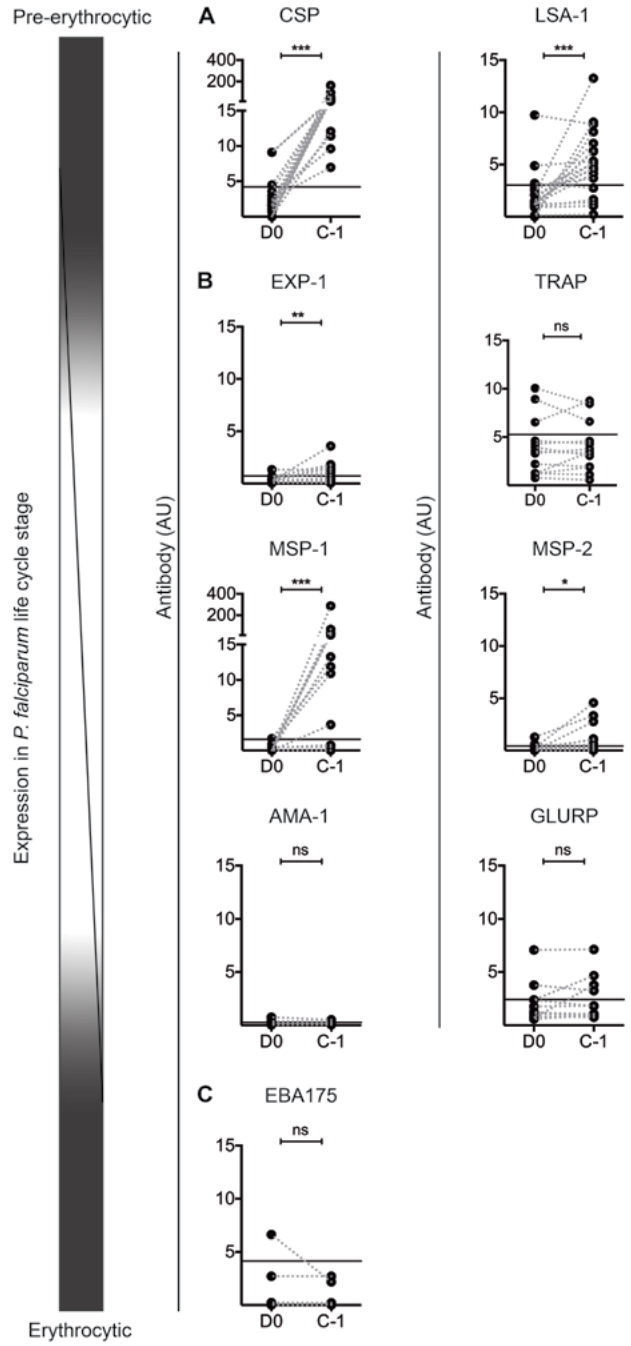

Figure 2 | Antibody responses against antigens representing different life-cycle stages of the malaria parasite
Antibody responses were determined before immunization (D0) and 19 to 21 weeks after the last CPS immunization (C-1) for volunteers immunized three times with bites from 15 infected mosquitoes (n=14 from Study A and n=5 from Study B). Responses are expressed as arbitrary units (AU) for malaria antigens representing different life-cycle stages of *P. falciparum* in the human host (schematically represented by illustration on the left): **A)** CSP and LSA-1 are expressed in pre-erythrocytic stages, **B)** while EXP-1, TRAP, MSP-1 and -2 and GLURP are present in liver and blood-stage parasites and **C)** EBA175 is exclusively expressed in *P. falciparum* infected erythrocytes. For Study B, responses were assessed for CSP, LSA-1, AMA-1 and MSP-1 only. Data are presented as ladder plots, with each dot representing an individual volunteer and dotted lines connecting values before and after immunization for each volunteer. The black line represents assay background levels (upper 99% Confidence interval of the mean of >40 malaria-naive samples tested). Differences between D0 and C-1 were analysed using Wilcoxon matched-pairs signed rank test. Significant differences are indicated by asterices * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), ns (not significant).

Antibody responses peaked after the third immunization, but then contracted in the following 15 weeks until C-1 (**Figure 3A**). Importantly, a different pattern was observed for the acquisition of MBC (**Figure 3B**): CSP- and MSP-1-specific MBC were also acquired in a stepwise fashion during CPS immunization, but for the majority of volunteers did not contract after the third immunization in the absence of parasite exposure, but instead remained stable or even increased over the more than 4 months until C-1. These patterns of CSP- and MSP-1-specific antibody and MBC acquisition were seen irrespective of immunization dose (**Figure 4A** and data not shown).

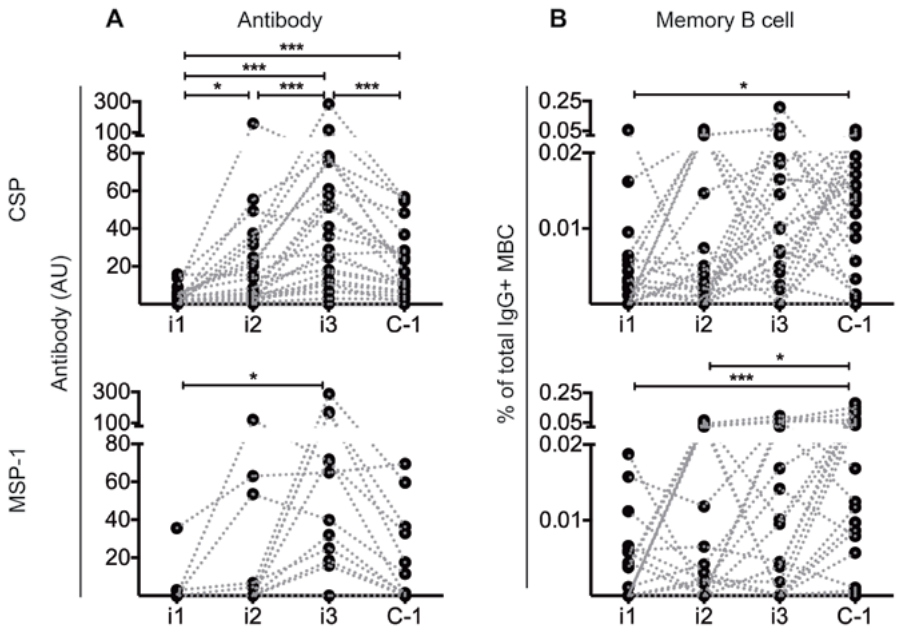

Figure 3 | Acquisition kinetics of antimalarial antibody and memory B cell responses
Antibody (**A**; in arbitrary units (AU)) and MBC responses (**B**; presented as % antigen-specific MBC of total IgG+ MBC) for CSP and MSP-1 were analyzed 28 days after the first (i1), second (i2) and third (i3) immunization and 19 weeks after i3 (C-1) for all CPS immunized volunteers of Study B (n=24). Data were corrected for the volunteers own background response at D0 and are presented as ladder plots, each dot representing an individual volunteer, and dotted lines connecting the different time points for each volunteer. Differences between the time points were analyzed by Friedman test with Dunn's multiple comparison post-hoc test. Significant differences are indicated by asterices: * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$).

The magnitude of CPS-induced humoral responses is a sensitive read-out of parasite exposure, but does not predict sterile protection

An important question is whether the magnitude of CPS-induced humoral responses to the antigens investigated reflects the degree of parasite exposure and whether they are predictive of sterile protection from challenge infection. We therefore compared immune responses in volunteers who received different immunization doses (Study B, **Figure 1**), and were subsequently either sterilely protected or not from mosquito challenge.

In volunteers protected from mosquito bite challenge, there was a trend for higher antibody responses to CSP with increasing amounts of immunizing infectious mosquito bites, consistent with the different doses of pre-erythrocytic parasites experienced (**Figure 4A**). CPS immunization with different numbers of infectious mosquito bites also lead to a different amount of blood-stage exposure measured by qRT PCR after the first immunization (**Figure 4B**). This is consistent with the dose-response relation for completed pre-erythrocytic and early erythrocytic development, which is in turn reflected in the induction of MSP-1 antibodies (**Figure 4A**). Blood-stage parasitemia was reduced after the second and completely absent after the third CPS immunization in volunteers subsequently sterilely protected from mosquito challenge (**Figure 4B**).

In the group receiving the lowest immunization dose (bites of 3x5 mosquitoes), half of the volunteers were unprotected from mosquito challenge. Unprotected volunteers had significantly higher antibody responses after the third immunization against both CSP and MSP-1 compared to protected volunteers receiving the same number of immunizing mosquito bites (**Figure 4C**). This was probably the result of the greater parasite exposure during the second and third immunization, reflected by the significantly higher blood-stage parasitemia compared to protected volunteers (**Figure 4D**).

Across all immunized volunteers in this dose de-escalation trial (Study B, **Figure 1**), magnitudes of CSP and MSP-1 antibody responses after the third immunization correlated with the cumulative blood-stage parasitemia to which volunteers were exposed over the course of the three immunizations (**Figure 4E**).

These data clearly demonstrate that the magnitude of the humoral response against CSP, LSA-1, AMA-1 and MSP-1 after CPS immunization does not predict protection from challenge infection, but accurately reflects parasite exposure (both the number of infectious mosquito bites and amount of blood-stage parasitemia) during CPS immunization.

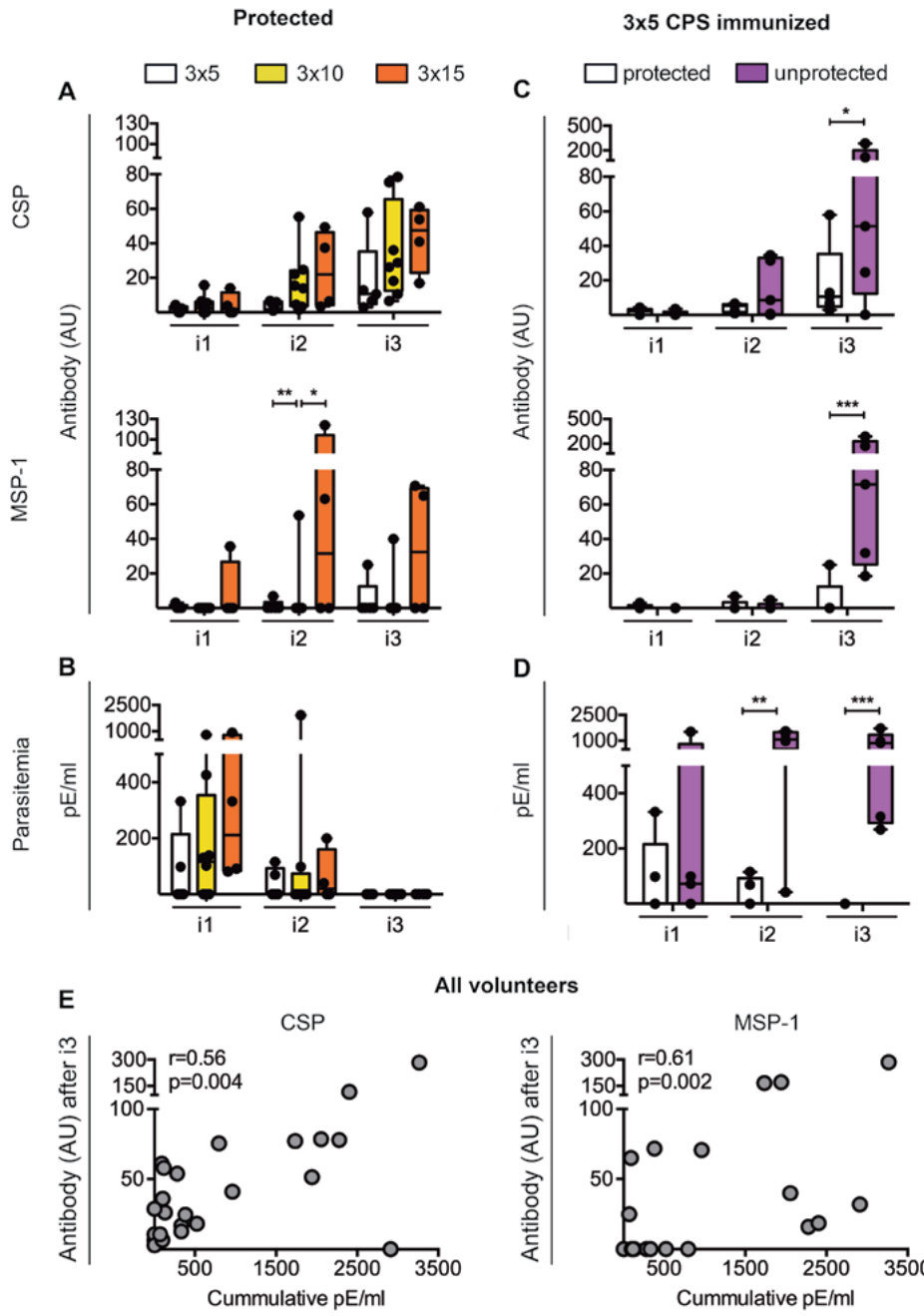

Figure 4 | Influence of parasite exposure during immunization on magnitude of the antibody response and protection status *(for legend see next page)*

Figure 4 | Influence of parasite exposure during immunization on magnitude of the antibody response and protection status *(see previous page)*
A/B CPS immunized volunteers of Study B protected from mosquito challenge (MC), receiving either 3x5 (white, n=5), 3x10 (yellow, n=8) or 3x15 (orange, n=4) bites from infectious mosquitoes during immunization. **A)** CSP and MSP-1-specific antibody levels (in arbitrary units (AU)) and **B)** blood-stage parasitemia (expressed as number of parasitized erythrocytes per ml blood (pE/ml, determined by quantitative RealTime PCR) were analyzed 28 days after first (i1), second (i2) and third (i3) CPS immunization. **C/D** 3x5 CPS immunization resulted in either protection from subsequent MC (white, n=5) or not (purple, n=5). **C)** CSP and MSP-1-specific antibody (in AU) and **D)** erythrocytic parasitemia (in pE/ml) 28 days after i1, i2 and i3 are displayed. **E)** Correlation between CSP and MSP-1 antibody responses (in AU) 28 days after immunization 3 and cumulative parasitemia (in pE/ml) over the course of all CPS immunizations for all Study B volunteers. All antibody-responses were corrected for the volunteers own background response at D0 and are presented as individual values (dots) and whisker box plots (box: median with 10-90 percentile; whiskers: min to max) and individual values (dots). Differences between groups over time were analyzed by repeated measures, mixed model two-way ANOVA with Bonferroni post-hoc test. Significant differences are indicated by asterices: * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$). Correlation was analysed by Spearman coefficient (r).

Boosting of humoral responses by challenge infection reflects exposure to different *P. falciparum* life-cycle stages.

Humoral immune responses acquired during CPS immunization were boosted dependent on parasite exposure by different challenge regimens and protection status (**Figure 5** and **Table S1**). A total of 22 immunized volunteers across both clinical trials were protected from mosquito challenge (Study A and B, **Figure 1**). Since they did not develop blood-stage parasitemia, these volunteers were only exposed to pre-erythrocytic parasites[13]. In line with this, antibody and MBC responses specific for CSP were boosted in these volunteers (**Figure 5A** and **Table S1**), but not responses specific for cross-and blood-stage antigens analyzed (**Table S1**). One volunteer, however, who was classified as protected from mosquito challenge by absence of thick smear-detectable parasites, was retrospectively found to have developed qRT PCR-detectable parasitemia (457 parasites/ml) at day 21 post-challenge just prior to presumptive drug-treatment[13]. This small exposure to blood-stage parasites was sufficient to induce a strong boost of MSP-1-specific antibody responses (white triangle in **Figure 5A**).
Seven CPS immunized volunteers were unprotected and thus experienced blood-stage parasitemia (as well as pre-erythrocytic parasites) after mosquito bite challenge infection (Study B, **Figure 1**). In line with exposure to a broad array of antigens, we found that antibody responses to the pre-erythrocytic antigens CSP and LSA-1 and the cross-stage antigens AMA-1 and MSP-1, as well as MBC responses to CSP, AMA-1 and MSP-1 were boosted in these volunteers (**Figure 5B** and **Table S1**).
Furthermore a group of nine CPS immunized volunteers was subjected to and

not protected from injection of parasitized erythrocytes (Study A, **Figure 1**), and thus did not experience sporozoites and liver-stage parasites. Antibody responses specific for the cross-stage antigens AMA-1, EXP-1, GLURP, MSP-1, MSP-2 and TRAP were boosted, but no boosting effect was observed for pre-erythrocytic antigens (**Figure 5C** and **Table S1**).

Interestingly, a single MC of malaria-naïve controls from Study A and B also induced CSP- and MSP-1-reactive antibodies in 80 and 90% of volunteers, respectively (**Table S2**), albeit at lower levels than after MC of primed CPS immunized volunteers. In the majority of volunteers a single infection was not sufficient to induce a detectable MBC response.

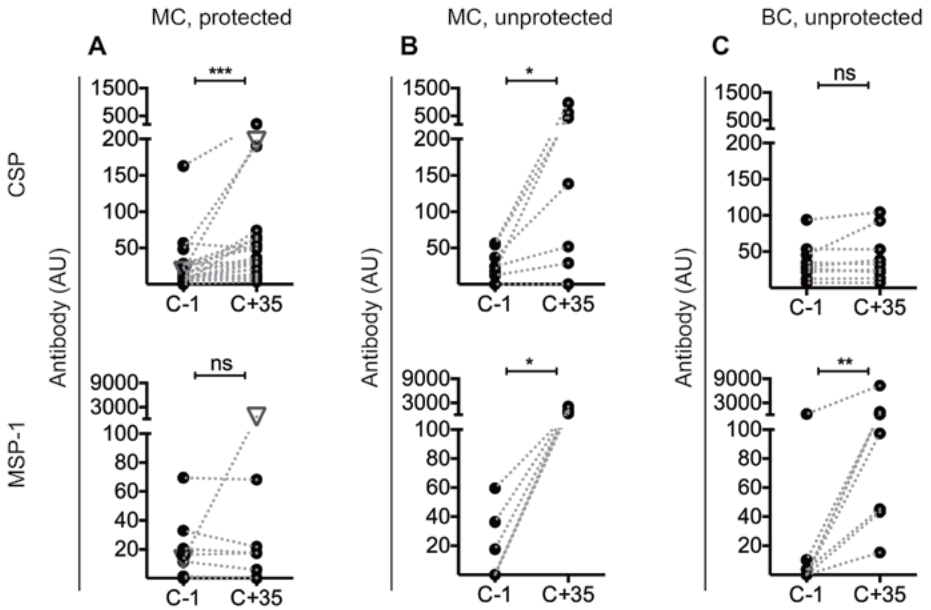

Figure 5 | Boosting of antibody levels in CPS immunized volunteers by challenge infection
Antibody levels (in arbitrary units (AU)), corrected for volunteers own background at D0) for CSP and MSP-1 in immunized volunteers were determined prior to (C-1) and 35 days following challenge infection (C+35). Each dot represents an individual volunteer for which values before and after challenge are connected by a dotted line. CPS immunized volunteers were grouped for analysis in: **A)** mosquito challenge (MC), protected (exposure to pre-erythrocytic parasites only), n=5 volunteers from Study A and n=17 volunteers from Study B; NOTE: White triangle: volunteer with qRT PCR detectable blood-stage parasitemia on day 21 post challenge. **B)** MC, unprotected (exposure to both pre-erythrocytic and blood-stage parasites), n=7 volunteers from Study B; **C)** blood-stage challenge (BC; exposure to erythrocytic parasites only), n=9 volunteers from Study A. Differences between time points were analyzed by Wilcoxon matched-pairs signed rank test. Significant differences are indicated by astericeswith * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$).

Discussion

In this study we provide the first comprehensive and side-by-side analysis of the kinetics and specificity of antibody and MBC responses generated in humans by CPS immunization and their association with sterile protection. We demonstrate that humoral responses, predominantly against pre-erythrocytic *P. falciparum* antigens, are efficiently generated in a stepwise fashion. The magnitude of these responses does not predict sterile protection from challenge infection, but is a sensitive marker of parasite exposure.

We show that CPS immunization induces strong humoral reactivity to pre-erythrocytic (CSP and LSA-1) and cross-stage (MSP-1) antigens, which is consistent with exposure mainly to pre-erythrocytic parasite antigens during CPS immunization. Exposure to erythrocytic-stage parasites is limited by chloroquine in this immunization regime; hence no humoral responses against AMA-1, GLURP and EBA175, which are mainly expressed during the erythrocytic stages of the parasite, were detectable. This recognition profile is in line with data from a previous CPS immunization study, where antibody reactivity towards CSP, but not schizont lysate or GLURP was observed in most volunteers[11]. Furthermore CSP and LSA-1 were also identified as the two predominantly recognized antigens by *P. falciparum* protein-microarray of CPS immunized volunteers[23].

The abundance of *P. falciparum*-specific antibodies and MBC after CPS immunization was associated with cumulative parasite exposure i.e. number of immunizations, immunization dose and blood-stage parasitemia experienced. Curiously, correlation of CPS-induced humoral responses with cumulative blood-stage exposure during immunization was not only observed for MSP-1, which is expressed in erythrocytic parasites, but also for CSP, which is often considered a sporozoite-specific antigen. However, it was shown that CSP-expression is not confined to the sporozoite stage, but continues until the late stages of liver-infection for *P. falciparum*[24] as well as for the rodent parasites *P. berghei* and *P. yoelii*[25,26]. Since antibody responses are a sensitive reflection of parasite exposure, this indicates that in protected volunteers CPS-induced protective immunity is targeting early pre-erythrocytic stages (sporozoites or early liver-stage parasites), thus reducing the liver-parasite load. Hence there is shorter/less exposure to the pre-erythrocytic antigen CSP, leading to lower anti-CSP responses in protected compared to unprotected volunteers.

Antibody and MBC levels expanded gradually after the first, second and third CPS immunization, a kinetic seen independently of the level of parasite exposure during immunization. Antibody titers declined in the absence of parasite exposure

between immunization and challenge, likely due to the physiological contraction of short-lived plasma cell responses, whereas MBC levels were largely stably maintained or even increased further. That MBC are much more long-lived than quickly waning serological responses, was recently also reported in African children no longer exposed to malaria[27]. Furthermore, multigravid Ghanaian women showed a much stronger and faster MBC response to a pregnancy-restricted *P. falciparum* erythrocyte membrane protein-1 than women in their first pregnancy, also indicating a successful generation and maintenance of humoral immune memory[28]. An intrinsic impairment in the generation of B cell memory as suggested previously in naturally malaria-exposed individuals[29,30], is not apparent in our study.

Challenge infection strongly boosted antibody responses acquired during immunization. This suggests an efficient differentiation of MBC into antibody secreting cells upon antigen-re encounter, even in volunteers experiencing patent blood-stage parasitemia during challenge infection. Therefore, low-grade blood-stage exposure during challenge infection in humans does not lead to an ablation of previously generated MBC or impaired survival of resulting antibody secreting cells, unlike previously shown for high erythrocytic parasitemias in rodent malaria models[31,32].

Boosting of humoral responses to the different *P. falciparum* antigens was dependent on exposure to different parasite life-cycle stages during challenge. MC boosted pre-erythrocytic and cross-stage responses, while exposure to blood-stage parasites boosted responses to cross-stage and erythrocytic antigens. Of note, the subunit vaccine candidate TRAP[33], often classified as a pre-erythrocytic *P. falciparum* protein, was also increased by blood-stage exposure during challenge, strengthening reports of TRAP or TRAP-like proteins being expressed in erythrocytic parasites[34,35]. We consistently found that MSP-1 antibody responses were strongly boosted in every single immunized volunteer experiencing both full liver-stage and patent erythrocytic parasitemia during challenge infection. That an increase in MSP-1 antibody levels is a reliable read-out even for low-level blood-stage exposure following B cell priming, in this case by CPS immunization, was illustrated strikingly in our study: One volunteer (classified as protected by the absence of parasitized erythrocytes on thick smear), was retrospectively shown by qRT PCR to have had sub-patent blood-stage parasitemia[13]. This small exposure to few *P. falciparum* infected erythrocytes for one day only lead to a strong boosting of the MSP-1 antibody response. By contrast, MSP-1 antibody levels in volunteers with qRT PCR-confirmed absence of blood-stage parasitemia

after challenge were not boosted. In turn, a lack of boosting of MSP-1-specific antibody responses after challenge infection may be used as an indicator of sterile pre-erythrocytic protection. Taken together, antibody and MBC responses observed in our study depended directly on the amount and life-cycle stage of parasite exposure during both CPS immunization and challenge.

The immunodominant antigens in our study, CSP and MSP-1, induced antibodies in the majority of volunteers already after a single CPS immunization or mosquito bite infection, and can thus be used as markers of recent parasite exposure. Indeed, CSP antibodies can be used to assess *P. falciparum* transmission dynamics in a naturally exposed population[36]. However, although humoral immune responses during CPS immunization are efficiently generated, there was no association between the magnitude of humoral responses to CSP, LSA-1, AMA-1 or MSP-1 or any combination of those antigens with protection from challenge infection. CSP-specific antibodies[37,38] were originally suggested as the mechanism of protection following immunization with irradiated sporozoites and have driven the development of CSP-based malaria vaccine candidate RTS,S. However, despite inducing high CSP-specific antibody responses, that can protect human liver-chimeric mice against *P. falciparum* sporozoite challenge[39], RTS,S only confers moderate protection against clinical and severe disease in children and infants in endemic areas[6,7]. The association of humoral responses against CSP with sterile protection therefore remains controversial[40-43]. Our results suggest that after whole sporozoite immunization under chemoprophylaxis, sterile pre-erythrocytic protection may be associated with recognition of novel, less immunodominant antigens. Encouragingly, a pilot protein-microarray study identified three novel antigens that were recognized exclusively by protected volunteers immunized with irradiated sporozoites[42]. Analysis of CPS immunization samples using an extended version of this protein-microarray will help to identify potential new protective antigens. Moreover, protection against malaria may not be associated with humoral responses to one antigen in isolation, but instead a panel of antigens[44]. In irradiated sporozoites-immunized volunteers, the cumulative response to 19 pre-erythrocytic antigens, rather than reactivity to an individual antigen, differentiated protected from unprotected volunteers[42].

Finally, while the magnitude of individual or combined humoral responses does not predict protection, the actual functional capacity of the resulting antibodies could do so. We have recently shown that antibodies induced by CPS immunization can reduce sporozoites traversal through hepatocytes *in vitro* as well as liver-stage infection and development *in vivo* in human liver-chimeric mice[45]. However, even

antibodies from sterilely protected volunteers were not able to fully prevent liver-stage infection[45]. In the dose de-escalation CPS immunization cohort, we have recently shown that sterile protection from challenge infection was associated with induction of higher levels of CD4 T cells with a cytotoxic phenotype (CD107a+)[20]. An integrated analysis of cellular and humoral responses both on antigen-recognition and functional level in the same group of volunteers will be crucial to solve the question of a potential immune correlate of sterile protection.

In conclusion, CPS immunization induced antibody and MBC responses mainly against pre-erythrocytic and cross-stage antigens of *P. falciparum*. Despite their efficient acquisition, the magnitude of acquired humoral immune responses to the set of immunodominant malarial antigens analyzed in this study did not predict sterile protection from challenge infection. Instead it proved to be a sensitive read-out for the degree and nature of parasite-antigen exposure during both immunization and challenge. The magnitude of acquired antigen-specific humoral responses alone can therefore not be used as a surrogate marker of protective efficacy. Other readouts and assessing responses to novel antigens are hence necessary to predict protection.

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Supporting information

Materials and Methods

Clinical trial: study design and sample collection

Study A[1]

Fourteen CPS immunized volunteers were assigned to receive a challenge with *P. falciparum*-parasitized erythrocytes (blood-challenge BC; n=9) or a sporozoite challenge by infected mosquito bites (MC; n=5). Ten additional volunteers were assigned to two control groups for either BC (n=5) or MC (n=5) (**Figure 1,** Study A). CPS immunization was conducted with three times 15 mosquitoes (eight mosquitoes infected with *P. falciparum* strain NF54 and seven mosquitoes infected with *P. falciparum* 3D7 (clone of NF54)). Twenty-one weeks following immunization (17 weeks after discontinuation of chloroquine prophylaxis), all volunteers were subjected to a challenge infection. MC was performed by exposure to bites of five *P. falciparum* 3D7-infected mosquitoes. BC was conducted by *intravenous* administration of 1962 viable 3D7 *P. falciparum*-infected erythrocytes. All ten control subjects (MC and BC) and all nine immunized BC volunteers showed parasitized erythrocytes on thick smear and qRT PCR, whilst the five immunized MC volunteers remained thick smear negative until 21 days after challenge and were then presumptively drug-treated.

Study B[2]

Twenty-nine volunteers were randomly assigned to four groups, three CPS immunization groups and one control group (**Figure 1**, Study B). CPS immunization was carried out using three different numbers of NF54-infected mosquitoes: Study subjects were subjected three times to bites of 15 infected mosquitoes (3x15; n=5), ten infected (and five uninfected) mosquitoes (3x10; n=9) or five infected (and ten uninfected) mosquitoes (3x5; n=10). Control subjects received bites from 15 uninfected mosquitoes (n=5). Therefore, data from 29 challenged subjects were available for analysis. Nineteen weeks following immunization (15 weeks after discontinuation of chloroquine prophylaxis), all volunteers were subjected to a MC challenge infection by bites of five *P. falciparum* NF54-infected mosquitoes. All five control subjects developed blood-stage parasitemia, as detected by thick smear and qRT PCR. Of the 24 immunized subjects, sterile protection from MC challenge (defined as blood-stage parasite negative by thick smear until day 21 after challenge) was observed in 4/5 volunteers in the 3x15 group, 8/9 in the 3x10

group and 5/10 of the 3x5 group. The remaining seven CPS immunized subjects were not protected, but did show a mean delay of 2.5 days in prepatent period of blood-stage parasitemia by both thick smear and qRT PCR compared to the control subjects. Twenty-one days after challenge, all volunteers remaining thick smear negative were presumptively drug-treated.

Sample collection

Sampling of citrate anti-coagulated peripheral blood for immunological analysis was performed at different time points in Study A and Study B using CPT vacutainers (Becton Dickinson). In Study A, samples were available from D0 (pre-immunization, before onset of chloroquine prophylaxis), day before challenge (C-1) and 35 days after challenge (C+35). In Study B, additional sampling was performed one month (28 days) after each of the three immunizations (**Figure 1**).

Malaria antigens

As representative for pre-erythrocytic antigens we assessed *P. falciparum* circumsporozoite protein (CSP) and liver-stage antigen 1 (LSA-1)[3]. Erythrocyte binding protein 175 (EBA175, purchased from Protein Potential, LLC (9800 Medical Center Drive, Suite A209 Rockville, MD 20852)) on the other hand is only expressed in erythrocytic parasites. Most proteins, however, despite being most highly abundant in blood-stage parasites, are also are expressed in late liver-stages (cross-stage antigen). The investigated cross-stage antigens were apical membrane antigen 1 (AMA-1)[4,5], exported protein 1 (EXP-1)[6], thrombospondin related anonymous protein (TRAP)[7] and the 19 kDa C-terminal region of merozoite surface protein 1 (MSP-1)[8] and 2 (MSP-2)[9] as well as glutamate rich protein (GLURP)[10].

Malaria antigen-specific memory B cell ELISpot assay

Mitogen stimulation

The generation of malaria-specific memory B cells (MBCs) was assessed by MBC ELISpot assay[11,12]. Cryopreserved PBMCs were thawed and 1x10^{6}cells/ml in RPMI containing 10% FCS, 100U/ml penicillin/streptomycin, 100mM HEPES, 50mM 2-β-Mercaptoethanol and 2mM L-Glutamine (all Invitrogen) were added to 25cm^2 cell culture flasks (Greiner). To promote development of MBCs into antibody-secreting cells (ASCs), PBMC were stimulated for five days at 37°C and 5% CO_2 with 50ng/ml Pokeweed Mitogen derived from Phytolacca americana (Sigma-Aldrich), 1:5000 Staphylococcus aureus Protein A, Cowan Strain (Sigma-Aldrich),

2.5µg/ml ODN 2006 (Type B CpG nucleotide-human TLR9 ligand; InvivoGen tlrl-2006 5'-TCGTCGTTTTGTCGTTTTGTCGTT-3') and 25ng/ml recombinant human IL-10 (PeproTech).

ELISpot assay

MultiScreen Filter PVDF Immobilon plates (MSIPS4510, Millipore) pre-treated with 35% Ethanol were coated overnight at 4°C with 10µg/ml monoclonal antibodies to human IgG (clones MT91/145; Mabtech) or 4µg/ml of one of the malaria antigens (all dilutions in PBS). Plates were washed thoroughly and blocked with 1% bovine serum albumin (BSA; Sigma-Aldrich) in RPMI for 2h at 37°C. 4x10^5 mitogen-stimulated PBMCs per well were seeded in quadruplicates into the malaria antigen-coated filter plates. Anti-IgG coated wells were seeded with 1200 or 4000 cells/well. All time points for one volunteer were measured on the same plate. Filter plates were incubated for 6h at 37°C, 5% CO_2. After washing, immobilized IgG antibody in the proximity of ASCs was detected using polyclonal goat anti-human IgG (Fcγ) alkaline phosphatase (1:1000 in PBST/0.5% FCS; Mabtech) overnight at 4°C. Plates were developed in the dark using alkaline phosphatase substrate BCIP/NBT (Mabtech), rinsed with water, left to dry and stored protected from light until reading using the CTL ImmunoSpot Reader (Cellular Technology Ltd.).

Background correction

Since pre-culture B cell proportions (percentage of total PBMCs) and post-culture ASCs (per million PBMCs) were highly variable between donors (median with range 5.97 % (1.12-18.32 %) and 31,792 (6625-138,125), respectively), we corrected for inter-individual differences by expressing malaria-specific MBCs as the percentage of total IgG+ MBCs. Group-background levels of MBCs were defined by upper 99% confidence interval of the mean of all time points pre-malaria exposure of all involved volunteers (CSP=0.0054, LSA-1=0.01, TRAP=0.0084, AMA-1=0.01, EXP-1=0.008, MSP1=0.01, MSP-2=0.01, EBA175=0.007, GLURP=0.009). If indicated, MBC responses are presented after individual background correction for each volunteer by subtraction of the average number of spots counted for the specific antigen prior to malaria exposure (immunized individuals D0, control volunteers C-1).

Measurement of malaria-specific plasma antibodies responses

ELISA

Concentrations of malaria antigen-specific antibodies were determined in citrate anti-coagulated plasma in relation to a pool of 100 sera from adults living in a highly endemic area in Tanzania (HIT serum[13]) by standardized enzyme-linked immunosorbent assay (ELISA). Polystyrene flat-bottom plates (NUNC™ Maxisorp, Thermo Scientific) were coated overnight at 4°C with 1µg/ml malaria antigen in PBS. After washing, plates were blocked with 3% BSA in PBS. Plasma samples were diluted in PBST/1% FCS and analyzed in duplicates. A four-point 1:2 dilution series was carried out for each sample. As a standard, duplicates of pooled HIT serum were included on every plate in a seven-point dilution series. The optimal dilution range was determined prior to the study for every antigen. All time points for one volunteer were measured on the same plate. Two-step detection was performed using biotinylated polyclonal goat anti-human IgG (Fcγ) (Mabtech; 1:1250) and streptavidin-conjugated horseradish peroxidase (HRP, Mabtech, 1:2000). All incubation steps from blocking to detection were carried out for 1h at 37°C in a humidified chamber. Plates were developed at room temperature using HRP substrate (Tetra-methyl-benzidine; tebu-bio); the reaction was stopped using 0.2M H_2SO_4. Spectrophotometrical absorbance at 450nm was measured using the Anthos 2001 ELISA plate reader.

Analysis

The standard curve of HIT serum was plotted on a logarithmic scale and fitted to a power trend line (R^2> 0.99), optical density (OD) measurements for each sample (average of duplicates that were no more than 15% different) were converted to arbitrary units (AU) in relation to HIT serum. For each antigen, undiluted HIT serum was defined to contain 100 AU of IgG directed against this antigen. Dilutions of test samples that did not fall within the linear part of the optical density range of the standard curve were excluded. Group-background levels (in AU) were defined by upper 99% confidence interval of the mean of all time points pre-malaria exposure of all involved volunteers (CSP=4.18, LSA-1=3.0, TRAP=5.25, AMA-1=0.27, EXP-1=0.72, MSP1=1.6, MSP-2=0.45, EBA175=4.16, GLURP=2.42). If indicated, values for malaria antigen-specific antibody responses are presented after individual background correction by subtraction of the average number of AUs for each volunteer for the specific antigen prior to malaria exposure (immunized individuals D0, control volunteers C-1).

Table S1 | Antibody and memory B cell responses in CPS immunized volunteers before and after challenge infection *(continued next page)*

Antibody (AU)[a] — Memory B cells (% of total IgG+ memory B cells)[a]

CPS immunized volunteers (Mosquito challenge, protected)[b]

	C-1		C+35				C-1		C+35			
	Median	Range	Median	Range	p-value[e]		Median	Range	Median	Range	p-value[e]	
CSP	10.72	0-163	30.87	3.8-210	0.0001	*	0.015	0-0.057	0.035	0-0.29	0.02	*
LSA-1	0.68	0-11.15	0.45	0-10.68	0.003	*	0	0-0.009	0	0-0.01	0.22	
AMA-1	0	0-0.09	0.008	0-0.12	0.19		0.004	0-0.02	0	0-0.029	0.17	
EXP-1	0.01	0.-0.99	0.202	0-1.5	0.13		0	0-0.002	0	0-0.007	0.75	
GLURP	0	0-0.045	0	0-1.05	1.00		0.002	0-0.016	0.005	0-0.007	0.63	
MSP-1	0	0-69.5	0	0-649	0.43		0.006	0-0.15	0.004	0-0.15	0.73	
MSP-2	0	0-2.05	0.02	0-2.8	0.25		0	0-0.005	0.009	0-0.015	0.25	
TRAP	0	0-0.45	0	0-0.73	0.5		0.002	0-0.012	0.002	0-0.011	0.88	
EBA175	0	0-0.004	0	0-0.004	1		0.002	0-0.004	0.002	0-0.005	1.00	

CPS immunized volunteers (Mosquito challenge, unprotected)[c]

	C-1		C+35				C-1		C+35			
	Median	Range	Median	Range	p-value[e]		Median	Range	Median	Range	p-value[e]	
CSP	24.4	0-56	138.5	0-965.4	0.03	*	0.017	0.006-0.04	0.046	0.023-0.11	0.02	*
LSA-1	6.06	1.02-16	11.18	99.8	0.02	*	0	0-0.031	0.009	0-0.015	0.44	
AMA-1	0.02	0-0.14	0.29	0.09-1.4	0.02	*	0.0001	0-0.0076	0.005	0-0.03	0.03	*
EXP-1	-	-	-	-	-		-	-	-	-	-	
GLURP	-	-	-	-	-		-	-	-	-	-	
MSP-1	0	0-59.6	979		0.02	*	0.068	0.012-0.18	0.22	0.07-0.5	0.02	*
MSP-2	-	-	-	-	-		-	-	-	-	-	
TRAP	-	-	-	-	-		-	-	-	-	-	
EBA175	-	-	-	-	-		-	-	-	-	-	

Table S1 | Antibody and memory B cell responses in CPS immunized volunteers before and after challenge infection *(continued)*

Antibody (AU)[a] Memory B cells (% of total IgG+ memory B cells)[a]

CPS immunized volunteers (Blood-stage challenge, unprotected)[d]

	C-1		C+35				C-1		C+35			
	Median	Range	Median	Range	p-value[e]		Median	Range	Median	Range	p-value[e]	
CSP	29.22	6.65-94	32.68	6.9-104.6	0.25		0.04	0-0.095	0.009	0-0.044	0.08	
LSA-1	5.21	0.44-9.1	4.43	0.72-19.0	0.36		0.006	0-0.069	0	0-0.026	0.22	
AMA-1	0	0-0.078	0.592	0.04-0.87	0.004	*	0	0-0.02	0.005	0-0.039	0.25	
EXP-1	0.3	0-3.32	3.02	0.27-7.76	0.004	*	0	0-0.016	0.004	0-0.04	0.38	
GLURP	0	0-3.08	0.26	0-5.15	0.008	*	0.003	0-0.016	0.002	0-0.01	0.31	
MSP-1	0	0-288.4	203.9	15.4-7308	0.004	*	0.005	0-0.019	0.003	0-0.058	0.38	
MSP-2	0.66	0-4.32	3.42	0-18.23	0.016	*	0	0-0.022	0	0-0.009	0.69	
TRAP	0	0-2.24	0.36	0-12.69	0.019	*	0.003	0-0.021	0	0-0.0024	0.03	*
EBA175	0	0-0.0067	0.002	0-0.032	0.44		0	0-0.024	0	0-0.006	1	

[a]Background corrected for each volunteer by subtraction of individual pre-immunization (D0) values
[b]Exposure to pre-erythrocytic stages only; n=5 from Study A and n=17 from Study B (for Study B, responses were assessed for CSP, LSA-1, AMA-1 and MSP-1 only)
[c]Exposure to both pre-erythrocytic and blood stages; n=7 from Study B
[d]Exposure to blood-stages only; n=9 from Study A
[e]Differences between time points were analyzed by Wilcoxon matched-pairs signed rank test. Significant differences are indicated by asterices: * (p<0.05), ** (p<0.01), *** (p<0.001).

Shading: pre-erythrocytic antigens (blue), Cross-stage antigens (green), Blood-stage antigen (red)

Table S2 | Antibody and memory B cell responses after a single *P. falciparum* mosquito bite infection

Antibody (AU)[a] Memory B cells (% of total IgG+ memory B cells)[a]

Controls (Mosquito challenge)[a]

	C-1		C+35				C-1		C+35			
	Median	Range	Median	Range	p-value[b]		Median	Range	Median	Range	p-value[b]	
CSP	1.13	0-24.72	12.39	0-40.59	0.004	*	0.002	0-0.026	0.002	0-0.036	0.04	
LSA-1	1.51	0.73-5.67	2.89	0.54-22.78	0.19		0.003	0-0.016	0.005	0.016	0.49	
AMA-1	0.16	0.05-0.68	0.14	0.07-0.69	0.77		0.004	0-0.018	0.002	0-0.012	0.46	
EXP-1	0.88	0.24-1.18	2	0.65-26.67	0.06		0.004	0-0.0094	0	0-0.068	0.63	
GLURP	1.38	0.78-4.19	1.05	0.83-5.46	0.81		0.004	0-0.016	0	0-0.009	0.88	
MSP-1	0	0-0.045	50.42	0-898.1	0.004	*	0.005	0-0.016	0.005	0-0.029	0.56	
MSP-2	0.2	0-1.36	0.69	0-7.94	0.5		0.003	0-0.0094	0.003	0-0.0075	0.88	
TRAP	3.32	0.82-7.62	3.29	0.32-6.14	0.44		0.005	0-0.008	0.001	0-0.013	0.88	
EBA175	0.06	0.02-0.15	0.09	0.01-0.09	0.44		0	0-0.0078	0.002	0-0.008	0.88	

[a]Exposure to *P. falciparum* infected mosquito bites (Controls n=5 from Study A and n=5 from Study B, for Study B, responses were assessed for CSP, LSA-1, AMA-1 and MSP-1 only)
[b]Differences between time points were analyzed by Wilcoxon matched-pairs signed rank test. Significant differences are indicated by asterices: * (p<0.05), ** (p<0.01), *** (p<0.001).

Shading: pre-erythrocytic antigens (blue), Cross-stage antigens (green), Blood-stage antigen (red)

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CHAPTER 5

Expansion of IgG+ B cells during mitogen stimulation for memory B cell ELISpot analysis is influenced by size and composition of the B cell pool

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Abstract

The memory B cell (MBC) ELISpot assay is the main technique used to measure antigen-specific MBCs as a readout of humoral immune memory. This assay relies on the ability of MBCs to differentiate into antibody-secreting cells (ASCs) upon polyclonal stimulation. The total number of IgG+ ASCs generated by mitogen-stimulation is often used as a reference point; alternatively antigen-specific MBCs are expressed as a frequency of post-culture peripheral blood mononuclear cells (PBMC) as a surrogate for absolute frequencies. Therefore, it is important to know whether IgG+ B cells are uniformly expanded during the preceding mitogen-culture as a true reflection of MBC frequencies *ex vivo*. We systematically compared B cell phenotype and proportions before and after mitogen-stimulation in cultures of 269 peripheral blood mononuclear cell samples from 62 volunteers by flow cytometry and analyzed the number of resulting ASCs. Our data show that the number of total IgG+ ASCs detected by ELISpot after mitogen-stimulation correlates with the proportion of IgG+ MBCs *ex vivo*, highlighting its general robustness for comparisons of study cohorts at group level. The expansion of total and IgG+ B cells during mitogen-stimulation, however, was not identical in all cultures, but influenced by size and composition of the *ex vivo* B cell compartment. The uncorrected readout of antigen-specific MBCs per million post-culture PBMCs therefore only preserves the quality, but not the magnitude of differences in the *ex vivo* MBC response between groups or time points, particularly when comparing samples where the B cell compartment substantially differs between cohorts or over time. Therefore, expressing antigen-specific cells per total IgG+ ASCs is currently the best measure to correct for mitogen-culture effects. Additionally, baseline information on the size and composition of the *ex vivo* B cell compartment should be supplied to additionally inform about differences or changes in the size and composition of the *ex vivo* MBC compartment.

Introduction

Humoral immunity is crucial to combat many infections and to provide protection against re-infection and after vaccination. Primarily, antibodies are used as readouts for humoral immunity since they can be easily measured by enzyme-linked immunoabsorbance assay (ELISA). Long-term humoral immune memory is, however, not only conveyed by antibody-producing long-lived plasma cells, but also relies on the efficient acquisition and maintenance of memory B cells (MBCs), who, upon antigen reencounter, can rapidly develop into antibody secreting cells (ASCs) to mount a strong secondary antibody response[1]. Circulating MBCs have low frequencies and are quiescent, i.e. do not secrete antibody. Two main methods addressing these challenges have been developed to quantify the magnitude of the circulating MBC response: Direct *ex vivo* quantification can be performed using flow cytometry upon labelling of MBCs with fluorescently labelled monomeric or tetrameric antigens[2-4]. Alternatively, MBC-secreted antibodies can be quantified (by Enzyme-linked ImmunoSpot assay (ELISpot) or ELISA) following a pre-activation step using mitogens to differentiate MBCs into ASCs[5]. This method is readily applicable to large numbers of samples and antigens (provided sufficient cells are available from each sample), without the need for fluorescent labelling, which can be challenging for individual antigens[6]. It has thus become the main readout used to measure antigen-specific MBCs in the context of infections, vaccinations or allergy[6-12]. A necessary underlying, but yet untested, assumption made when using the MBC ELISpot to quantify antigen-specific responses is that MBCs are differentiated into ASCs at a fixed ratio[13]. Antigen-specific MBC responses measured using this technique are either reported as the number of ASC per million post-culture peripheral blood mononuclear cells (PBMC), or as percentage of total ASCs. Expressing antigen-specific cells as a proportion of ASC-differentiated MBC corrects for variation in both total MBC precursor frequencies and potential differences in expansion during mitogen-culture between donors. Reporting antigen-specific ASCs as per million post-culture PBMCs is mostly used to get insights into the absolute frequency of antigen-specific MBCs. This is done to also take into account inter-individual variations in total MBC frequencies particularly when comparing across age groups[12,13] that differ not just in their antigen-experience but also the size and composition of the (memory) B cell compartment[12,14]. This readout, however, does not correct for expansion and potential skewing of the MBC compartment during mitogen-culture. It is therefore important to know (i) whether B cells are indeed consistently expanded during mitogen-culture, (ii) which factors influence

this expansion, and (iii) whether the total number of Ig-secreting cells generated truly reflects MBC frequencies *ex vivo*. One study has addressed this last point for antigen-specific MBCs[4], but the readout by ELISA did not allow comparisons of ASCs following mitogen-stimulation and *ex vivo* MBCs on the single-cell level. A follow-up study demonstrated that the two possible readouts after culture with mitogens, limiting dilution ELISpot/ELISA and bulk culture ELISpot, correlated for two out of the three antigens analyzed[6], supporting the use of the cheaper and less time consuming bulk ELISpot. However, none of these studies investigated the effect of mitogen-culture on B cell expansion, which would have affected both post-culture read-outs equally.

In this study, we therefore investigated both the consistency of B cell expansion during mitogen-culture as well as the robustness of this assay to detect total IgG+ ASCs that correspond to *ex vivo* IgG+ MBC frequencies, by systematically comparing B cell phenotype and proportions before and after mitogen-stimulation in a large number of human PBMC cultures.

Materials and Methods

Peripheral blood mononuclear cells

To enable systemic analysis of B cell expansion and ASC generation in a large number of mitogen-stimulated cultures, we took advantage of 269 individual PBMC samples that were collected longitudinally from 62 healthy adult volunteers (age range 18–32 years) enrolled in two clinical trials conducted at the Radboud university medical center (Nijmegen, The Netherlands) and the Leiden University Medical Centre (Leiden, The Netherlands) between March 2011 and April 2012. Both studies received approval by the Central Committee for Research Involving Human Subjects of The Netherlands (CCMO; NL34273.091.10 and NL33904.091.10) and volunteers enrolled in the studies provided written informed consent. The study team complied with the Declaration of Helsinki and Good Clinical Practice including monitoring of data. The trials are registered at ClinicalTrials.gov, number NCT01236612 and NCT01218893.

In Study A[15], volunteers received a prophylactic dose of chloroquine for three months and bites from 3x15 *Plasmodium falciparum*-infected mosquitoes (Chemoprophylaxis and sporozoites, CPS), and were subjected to challenge with *P. falciparum*-infected red blood cells (n= 9) or infective mosquito bites (n= 5). Ten volunteers received only chloroquine prophylaxis and then a single *P. falciparum* challenge either with *P. falciparum*-infected red blood cells (n= 5) or mosquito-bites (n= 5). In Study B[16] volunteers undergoing chloroquine chemoprophylaxis were immunized with bites from either three times 15 (n= 5), ten (n= 9) or five (n= 10) *P. falciparum*-infected mosquitoes, or not-immunized (n= 5). All immunized and control subjects in this study were subjected to mosquito-bite challenge. Sampling of citrate anti-coagulated peripheral blood for immunological analysis was performed at different time points using CPT vacutainers (Becton Dickinson). In Study A and B, samples were available for each volunteer from D0 (preimmunization, before onset of chloroquine prophylaxis), day before challenge (C-1) and 35 days after challenge (C+35). In Study B, additional samples were available from one month (28 days) after each of the three immunizations (for CPS immunized volunteers only) as well as 140 days after challenge (C+140). Additionally, we included single samples from 9 healthy, Dutch adult malaria-naıve volunteers. At none of the blood collection time points for this study was any of the volunteers infected with *P. falciparum.*

PBMCs were isolated by density centrifugation. Following four washes in phosphate buffered saline (PBS), PBMCs were cryopreserved at $2x10^7$ cells/ml

in ice-cold fetal calf serum (FCS; Gibco)/10% DMSO (Merck) using Mr. Frosty freezing containers (Nalgene). PBMC samples were stored in vapour phase nitrogen.

Mitogen-stimulation

Differentiation of MBCs into ASCs was initiated in bulk PBMC cultures based on a previously established protocol[5], using a stimulation cocktail composed of pokeweed mitogen (PWM), *Staphylococcus.aureus* Cowan I protein A (SAC) and CpG. IL-10 was added to the stimulation mix since a previous study showed that this enhanced the efficiency of MBC into ASC differentiation by more than 9-fold[6]. Briefly, PBMCs were thawed for 30sec in a 37°C water bath and cold RPMI medium was immediately added drop wise. After washing, the cells were re-suspended in RPMI containing 10% FCS, 100U/ml penicillin/streptomycin, 100mM HEPES, 50mM 2-β-Mercaptoethanol and 2mM L-Glutamine (all Invitrogen) and counted. $1x10^6$ cells/ml were added to $25cm^2$ cell culture flasks (Greiner). Culture medium was supplemented with 50ng/ml PWM lectin derived from Phytolacca americana (Sigma-Aldrich), 1:5000 Protein A from *Staphylococcus aureus*, Cowan Strain (Sigma-Aldrich), 2.5µg/ml ODN 2006 (Type B CpG nucleotide-human TLR9 ligand; InvivoGen tlrl-2006) and 25ng/ml recombinant human IL-10 (PeproTech) and incubated at 37°C, 5% CO_2 for 5 days.

Memory B cell ELISpot

MultiScreen Filter PVDF Immobilon plates (MSIPS4510, Millipore) were pre-wetted with 35% Ethanol, rinsed twice with PBS and coated with 10µg/ml monoclonal antibodies to human IgG (clones MT91/145; Mabtech) diluted in PBS. After overnight incubation at 4°C, plates were washed (1x PBS/0.05% Tween 20 (Sigma-Aldrich, PBST), 3x PBS) and blocked with 1% bovine serum albumin (BSA; Sigma-Aldrich) in RPMI for 2 h at 37°C. Cells were harvested from the flasks, counted and seeded in quadruplicates at both 1200 and 4000 cells per well. Filter plates were incubated for 6h at 37°C, 5% CO_2 with minimized ambient vibration. After washing (4x PBS, 4x PBST), immobilized IgG was labeled using polyclonal goat anti-human IgG (Fc**γ**) alkaline phosphatase (1:1000 in PBST containing 0.5% FCS; Mabtech) overnight at 4°C. Following thorough washing (4x PBST, 4x PBS, 3x distilled water), alkaline phosphatase substrate BCIP/NBT (Mabtech) was added and the filter plates were kept in the dark until fully developed. Developed plates were rinsed with water, left to dry and stored protected from light until reading using the CTL ImmunoSpot Reader (Cellular Technology Ltd.). The mean number of IgG+ ASCs in quadruplicate wells was calculated per million PBMCs and averaged between the two cell concentrations. To check for assay background some wells on every plate received only medium instead of cells (no background detected) or $4x10^5$ cells were added to wells coated with 5 µg/ml the irrelevant antigen Bovine Serum Albumin (BSA; background: 0.33 spots/well).

Flow cytometry analysis

IgG+ B cells and B cell proportions were analyzed prior to (*ex vivo*; stain 1; **Figure S1**) and after mitogen-culture (stain 2; **Figure S2**). B cell subsets *ex vivo* were distinguished as described previously[17]. For post-mitogen-culture phenotyping we chose a different staining panel for the following reasons: (i) mitogen-stimulation results in a down-regulation of CD19 expression, necessitating the inclusion of CD20 to identify B cells. (ii) we aimed to assess proliferation after stimulation, thus including Ki67. (iii) in previous experiments we found that CD21 as well as IgD expression are down-regulated during culture of B cells, while CD38 expression is strongly up-regulated in the majority of B cells by mitogen-stimulation (**Figure S3**), thus preventing us to use the same gating strategy to distinguish different B cells subsets after 5 days of culture.

$5x10^5$ to $1x10^6$ cells per stain were transferred to 96 wells V-bottom plate (Sarstedt), washed with PBS and incubated for 30 min on ice with 50µl viability dye diluted in PBS (fixable viability dye eFluor 450, eBioscience (stain 1); aqua LIVE/DEAD fixable dead cell stain, Invitrogen (stain 2)). Cells were washed twice with staining buffer (PBS/0.5% BSA), and stained with 50µl antibody cocktail diluted in staining buffer for 30min at room temperature (RT). Antibodies used for surface staining are listed in Table 1 and Table 2. After another wash, cells were resuspended in 50µl fixation/permeabilization buffer (eBioscience), incubated for 30min at 4°C, and washed with 150µl permeabilization buffer (eBioscience). For intracellular staining, cells were incubated for 30min at RT with 50µl antibody cocktail (stain 2; Ki67 AlexaFluor 647 (B56); BD Biosciences) diluted in permeabilization buffer (eBioscience). Cells were washed with permeabilization buffer and re-suspended in 200µl PBS/1% paraformaldehyde. 1 to $2x10^5$ events per sample were acquired on a Cyan ADP 9-colour flow cytometer (Dako/Beckman Coulter) and analysis performed using FlowJo v9.2 software.

Statistical analysis

Data were analyzed using GraphPad Prism v5. B cell proportions *ex vivo* versus post-culture were compared by Wilcoxon matched pairs signed rank test. Skewing of distributions was tested by D'Agostino and Pearson omnibus normality test. Relationships between B cell proportions, expansion (fold change; calculated

by dividing for each sample the absolute number of total (or IgG+) B cells post mitogen-culture by the absolute number of total (or IgG+) B cells *ex vivo*) and ASCs were analyzed by non-parametric Spearman correlation. Correlation coefficients listed are for PBMC samples of the 62 volunteers at baseline, i.e. a time point when all of them where malaria-naıve and none of them was undergoing chloroquine prophylaxis. Correlation coefficients obtained for baseline samples only were comparable to those obtained when analyzing all 269 cultures from all collected time points.

Results

Applying both flow cytometry and ELISpot, we conducted a systematic large-scale analysis of 269 PBMC samples (derived from 62 donors sampled over 1–7 time points) before and after mitogen-stimulation (**Figure 1A**). We firstly determined the *ex vivo* composition of the B cell compartment (for subset definitions see **Table 3** and **Figure S1**), and particularly of IgG+ MBCs, from which IgG+ ASCs originate. Upon thawing of cryopreserved PBMCs, B cells made up 5.97% (median, interquartile range (IQR) 4.26–8.25%) of PBMCs, and 7.86% (median; IQR 5.69– 11.5%) of these B cells were IgG+. The IgG+ B cell compartment was largely composed of CD38lowIgD-CD102 MBCs (**Figure 1B**), dominated by CD21+CD27+ classical MBCs (cMBC; median 63.4%, IQR 55.9–67.8%), followed by CD21+ CD27- MBCs (median 20.3%, IQR 15.4–24.6%). Three other MBC populations constituted 3.3–5% each of the IgG+ B cell compartment, i.e. CD21-CD27- atypical MBCs (atypMBC), CD21-CD27+ activated MBC (actMBC) and IgD+CD21+CD27+ non-switched MBCs (nsMBC). With the exception of a slight dip in total B cells four weeks after the last immunization, both the size of the total and IgG+ circulating B cell compartment, and the proportions of the different MBC subsets remained constant over time in the 53 donors from which multiple samples were available (**Table S1** and **Table S2**), highlighting that the interventions the volunteers underwent in the clinical trials had no biasing effect on the samples analyzed. Recovery of total seeded PBMC after 5 days of culture in the presence of mitogens was 95.45% (median, IQR 82.9–112.1%). Consistent with 90.4% (median; IQR 87.5–92.1%) of B cells proliferating at day 5 (based on Ki67 staining, **Figure S3**), mitogen-stimulation successfully increased the proportion of total B cells (**Figure 1C**, $p<0.0001$) and IgG+ B cells within recovered PBMCs in all cultures (**Figure 1D**, $p<0.0001$). While the absolute number of both total and IgG+ B cells prior to culture correlated with their number after culture, this relationship was stronger for total B cells (Spearman $r= 0.87$, $p<0.0001$; **Figure 2A**), than for IgG+ B cells ($r= 0.67$, $p<0.0001$; **Figure 2B**). For total B cells, the median fold increase in absolute numbers over 5 days of culture was 6.04 (IQR 5.0–7.74; $p<0.001$), and this increase in total B cell numbers during mitogen-culture was not normally distributed (D'Agostino and Pearson omnibus normality test; skewness 1.06, kurtosis 2.04). Since all mitogen-cultures were performed under identical conditions, the most likely confounding factor was the variation in the starting proportion of total B cells (median 5.97%, range 1.1–18.3%). Indeed, we found that expansion of B cells during mitogen-culture was variable and dependent of B cell proportions before stimulation with an inverse and non-linear (exponential

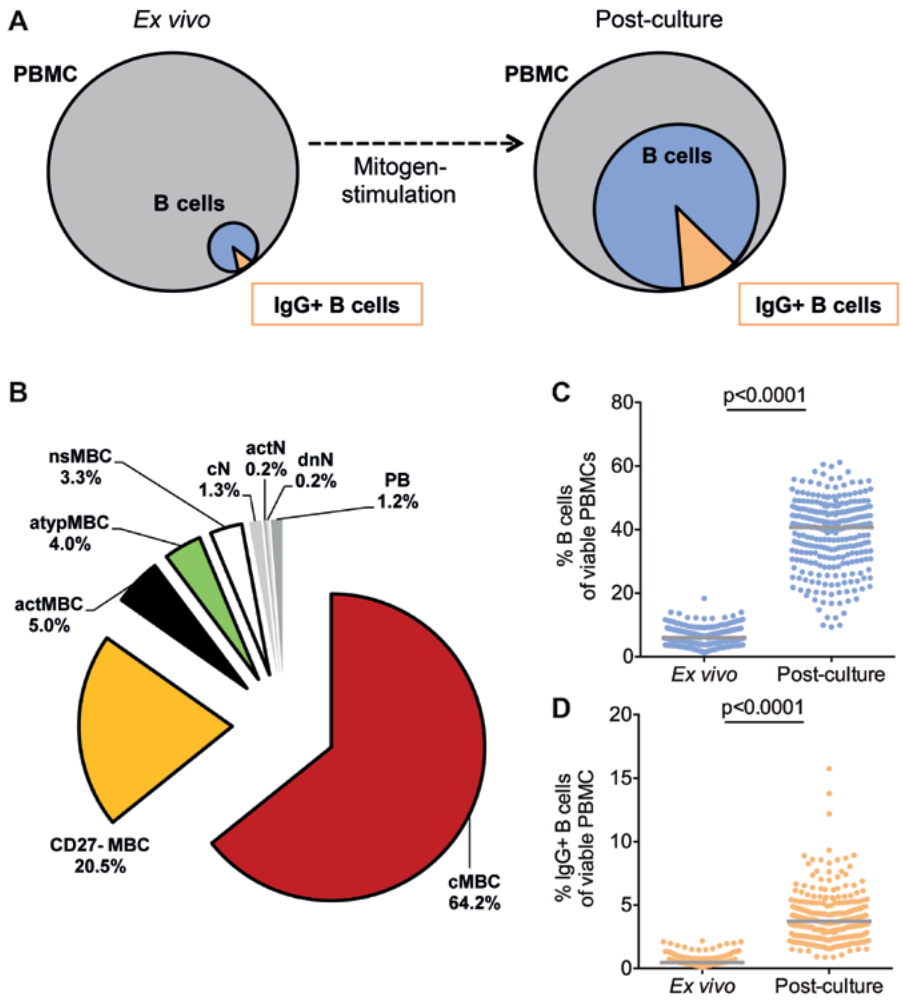

Figure 1 | Expansion of total and IgG+ B cells following mitogen-stimulation
Panel **A)** schematically illustrates the cellular composition of PBMCs directly *ex vivo* and after mitogen-culture. The grey circle represents total PBMCs, the blue circle all CD19+ B cells and the orange triangle IgG+ B cells. **B)** The composition of the IgG+ B cell compartment *ex vivo* was analyzed by flow cytometry and is depicted as median proportions of individual B cell subsets within total CD19+ B cells for baseline samples of 62 donors. The individual B cell subsets were subdivided based on IgD, CD38, CD10, CD21 and CD27 expression and include five memory B cell subsets: classical MBCs (cMBCs, red), CD27- MBCs (yellow), activated MBCs (actMBC, black), atypical MBCs (atypMBC, green) and non-switched MBCs (nsMBC, white). Depicted in shades of grey are plasma blasts (PB), activated naive B cells (actN), classical naive B cells (cN) and double-negative naive B cells (dnN). Panels **C–D** show *ex vivo* and post-culture proportions of total CD19+ B cells (**C**, blue dots) and IgG+CD19+ B cells (**D**, orange dots) within viable PBMCs. Black lines indicate the median. Dots show all 269 cultures. Differences between *ex vivo* and post-culture samples were compared by Wilcoxon matched pairs signed rank test.

decay) relationship between the *ex vivo* proportion and the fold increase in both their percentage (r= 20.69, p<0.0001, data not shown) and absolute number (r= 20.38, p= 0.023; **Figure 2C**) during culture. The largest expansion was found in cultures with the lowest starting proportions of total B cells. We next addressed whether mitogen-culture affected the composition of the B cell compartment. Overall, proportions of IgG+ cells within the B cell pool only increased slightly (pre- versus post-culture: median with IQR 7.68% (5.7–11.5%) and 10.3% (7.7–12.8%), respectively). However, as for B cells within total PBMCs, we also found that the *ex vivo* percentage of IgG+ cells strongly influenced their expansion within the B cell pool, with a strong inverse and skewed correlation (**Figure 2D**, r= 20.65, p<0.0001) between their starting proportions and fold increase during culture (median fold change post-culture/*ex vivo* 1.22, IQR 0.88–1.6; skewness 7.1; kurtosis 58.1). These effects of pre-culture size and composition of the B cell pool on the efficiency of mitogen-driven expansion also affected the increase in IgG+ B cells, with a strong inverse correlation between starting proportions and the increase in their percentage (r= 20.76, p<0.0001, data not shown) or absolute numbers (r= 20.70, p<0.0001; **Figure 2E**): This was particularly evident in cultures with a low proportion of starting IgG+ B cells (<0.5% of PBMCs; 155/269), where expansion was higher (median fold increase 9.3), more variable (IQR 6.7–13.4) and skewed (skewness 5.6, kurtosis 34.7) than in cultures with higher pre-culture proportions of IgG+ B cells (≤0.5% of PBMC; 114/269; median fold increase 5.3; IQR 4.4–6.7; skewness 2.2, kurtosis 7.8). Finally, expansion of IgG+ B cells was higher than that of IgG**-** B cells (**Figure 2E**, **F**; median fold increase 7.16 vs 5.86; p<0,0001), and expansion of IgG+ B cells more strongly influenced by their starting proportion (skewness 6.9, kurtosis 55.9; Spearman r= -0.70) than IgG**-** B cells (skewness 0.99, kurtosis 1.7; Spearman r= -0.29).

Taken together, differences in both size and composition of the *ex vivo* B cell compartment do directly influence the outcome of mitogen-stimulation cultures. When comparing the number of IgG+ B cells determined by flow cytometry to the number of IgG+ ACSs detected by ELISpot, both per million PBMCs post-culture, we found that the number of IgG+ B cells, although in a similar range, was slightly but significantly higher than that of IgG+ ASCs (**Figure 3A**; p= 0.003). This suggests that the majority of IgG+ B cells expanded during mitogen-culture also differentiate into ASCs. There was, however, only a relatively weak correlation between the proportion of IgG+ B cells and IgG+ ASCs after mitogen-culture (Spearman r= 0.48, p<0.0001; **Figure 3B**), showing that for a large number of cultures, staining of post-culture PBMCs for surface IgG will overestimate the

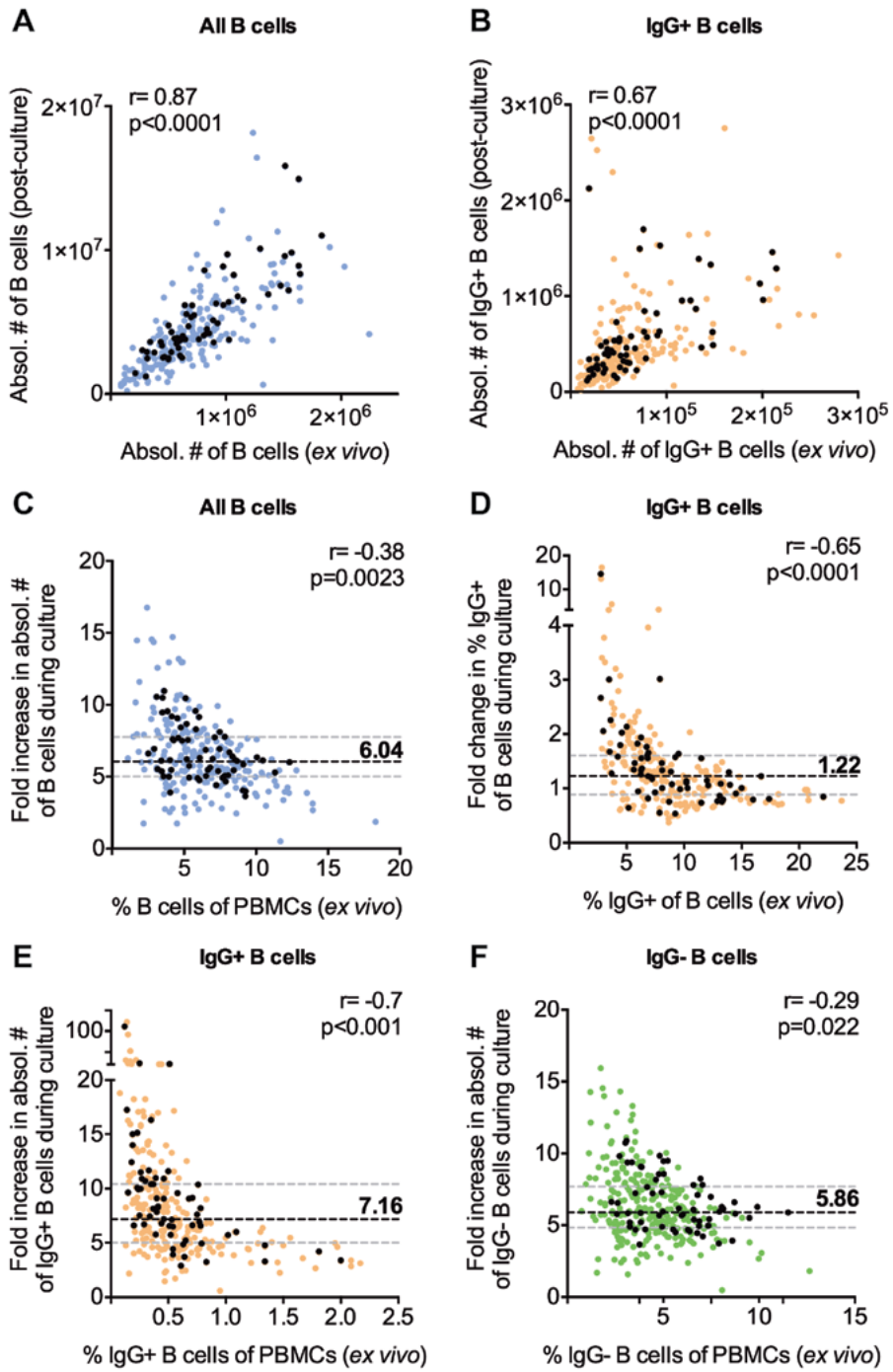

Figure 2 | Size and composition of the B cell compartment *ex vivo* influences B cell expansion during culture *(for legend see next page)*

Figure 2 | Size and composition of the B cell compartment *ex vivo* influences B cell expansion during culture *(see previous page)*
Flow cytometry analysis was performed to determine proportions and subsequently calculate absolute numbers of **A)** total CD19+ B cells (blue dots) and **B)** IgG+CD19+ B cells (orange dots) *ex vivo* and post-culture. *Ex vivo* proportions of **C)** total CD19+ B cells (blue dots) within viable PBMCs were plotted against the fold change in their absolute numbers, and **D)** IgG+ B cells (orange dots) within total CD19+ B cells were plotted against the fold change in their proportion within the B cell compartment. **E)** Proportions of IgG+CD19+ B cells (orange dots) and **F)** IgG-CD19+ B cells (green dots) within viable PBMCs were plotted against the fold increase in their respective absolute numbers post-culture compared to *ex vivo*. Colored dots show cultures from all 269 stimulated samples (3–7 time points per volunteer), while black dots show the cultures from only the 62 baseline samples (1 for each individual volunteer). The black dashed line indicates the median fold change (with value), grey dotted lines represent the upper and lower limit of the interquartile range. Spearman r and p values are shown for analysis of baseline samples (black dots) from the 62 donors assessed.

potential of these cells to secrete IgG, as detected by ELISpot. When IgG+ ASC numbers (per million PBMCs) were compared instead to the *ex vivo* proportion of IgG+ cells within PBMCs prior to culture, this correlation improved (r= 0.55, p<0.0001; **Figure 3C**), showing that IgG+ ASCs detected by MBC ELISpot are overall a good estimate of *ex vivo* MBC frequencies. Moreover, when taking out variations in the size of the B cell pool by analyzing *ex vivo* IgG+ proportions within the B cell compartment, the correlation with IgG+ ASC numbers post-mitogen-culture improved further (r= 0.65, p<0.0001; **Figure 3D**). This correlation was the same for IgG+ cMBCs, which constitute the largest proportion of the IgG+ B cell pool (r= 0.65, p<0.0001). IgG+ ASC numbers post-culture further correlated with the *ex vivo* proportions of IgG+ CD27- MBCs (r= 0.51, p<0.0001), the second largest population within IgG+ MBCs, but not for IgG+ atypMBCs (r= 0.24, p= 0.06). Of note, across all baseline samples of 62 volunteers, the ratio of atypMBCs/cMBCs correlated inversely with the proportion of IgG+ cMBCs *ex vivo* (r= -0.54, p<0.0001), but correlated positively with the expansion of IgG+ B cell numbers during mitogen-culture (r= 0.49, p<0.0001, data not shown).

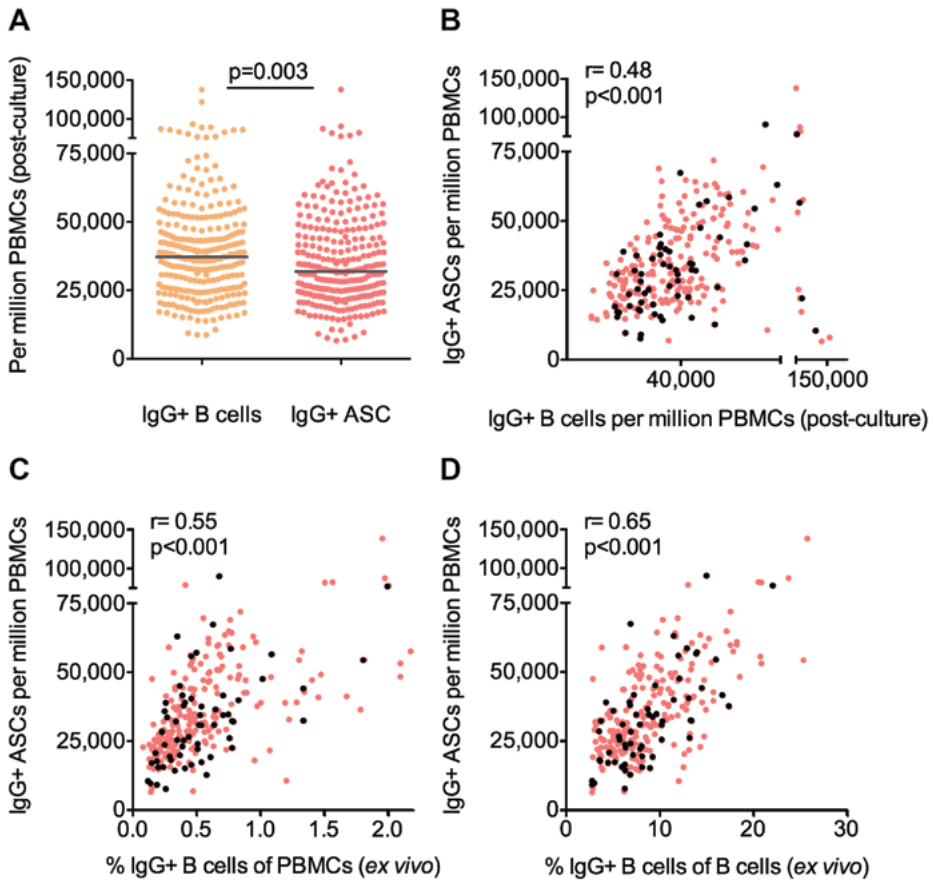

Figure 3 | Relationship between post-culture IgG+ ASC numbers and *ex vivo* or post-culture IgG+ B cell proportions

After 5 days of mitogen-culture, proportions of IgG+ B cells were analyzed by flow cytometry (orange dots) and IgG+ ASCs were quantified by ELISpot (red dots) and expressed as per million post-culture PBMCs **A)**. Black lines indicate the median. Post-culture **B)** and *ex vivo* proportions **C)** of IgG+ cells within PBMCs, and *ex vivo* proportions of IgG+ cells within CD19+ B cells **D)** were plotted against the number of IgG+ ASCs per million PBMCs. Red dots show cultures from all 269 stimulated samples (3–7 time points per volunteer), while black dots show the cultures from only the 62 baseline samples (1 for each individual volunteer).

Discussion

The MBC ELISpot assay is the most widely used method to quantify MBC responses in addition to plasma antibodies as an independent readout of humoral immune memory[6-12,18]. A basic assumption when using the MBC ELISpot assay to estimate the frequency of antigen-specific MBCs is that in the preceding mitogen-culture, all MBCs are expanded and differentiate into ASCs in a constant manner or at a fixed ratio, although this has not been examined to date[13]. We show here that the expansion of total and IgG+ B cells during culture is not constant, but instead influenced by both the size and composition of the *ex vivo* B cell compartment, which vary widely between individuals. At group level, the number of IgG+ ASCs detected by ELISpot after mitogen-stimulation indeed reflects the proportion of IgG+ MBCs *ex vivo*, in particular for the two major populations of IgG+ MBCs, i.e. cMBCs and CD27**-** MBCs. This good correlation between total input (*ex vivo* IgG+ MBC) and output (IgG+ ASC) is in line with a previous small-scale study on TTX-specific MBCs[4] and highlights the general robustness of this assay to compare antigen-specific MBC frequencies on group level (e.g. between different cohorts). This relationship was found across cultures from 62 volunteers with a wide range of *ex vivo* IgG+ MBC frequencies. We also observed, however, that the expansion of both B cells and IgG+ B cells during culture showed an inverse correlation with the size and composition of the pre-culture B cell compartment. This was particularly evident for IgG+ MBCs. As a result, when total or antigen-specific MBCs are expressed as a proportion of total post mitogen-culture PBMCs to estimate their frequency, this introduced bias into the readout, rather than allowing to take into account inter-individual variations in total *ex vivo* MBC frequencies[13]. The reason underlying this inverse relation between the *ex vivo* proportion of total or IgG+ B cells with their respective increase in absolute numbers during culture remains unknown. One possibility is that the T cell compartment is relatively larger in cultures with lower B cell proportions, and thus more T cells are available for cytokine production upon PWM-stimulation, providing more favorable expansion conditions than in cultures with higher B cell and lower T cell numbers. This factor would influence total B cell expansion whenever mitogen-stimulation is performed in bulk PBMC cultures. It does not, however, explain the skewing for IgG+ B cells within the B cell compartment, since cultures with the lowest total B cell proportion where not those with the lowest proportion of IgG+ B cells within the B cell compartment and vise versa. Alternatively, assuming the same expansion efficiency in all samples, IgG+ B cells in samples with a high starting proportion might die in culture due to crowding, limited nutrients or toxin build up, whereas

IgG+ B cells in samples with a low starting proportion can continue to proliferate and differentiate until the end of the 5 day culture. While this again may be true for the B cell compartment as a whole, we consider it unlikely to be the sole reasons for the strong inverse relationship between starting proportion and expansion of IgG+ B cells: Firstly, the strong skewing effect was also observed within the B cell compartment for IgG+ B cells. Secondly, albeit 10 times more numerous, starting proportion of IgG- B cells (containing mostly naive B cells and a few IgA+ or IgM+ MBCs) had only a very weak influence on their expansion in culture. This suggests that other reasons underlie these differences in expansion efficiency of IgG+ B cells. The skewing effect of B cell frequency and composition particularly affected cultures with a low proportion of *ex vivo* IgG+ MBCs, which were expanded with a greater magnitude and variation than those in culture with high starting proportions. One potential explanation for this observation is that expansion of IgG+ MBCs might also be enhanced by direct cell-cell contact of IgG+ B cells with PWM-activated T cells (for instance via CD40- CD40L[19]): when the IgG+ B cell population is smaller to begin with, the likelihood for direct cell-cell contact may be lower, and interactions thus more random, than when IgG+ B cells are more frequent. In line with a greater variation in expansion, when *ex vivo* IgG+ MBC proportions were plotted against post-culture IgG+ ASCs, the spread was wider and the correlation coefficient weaker for cultures with lower starting proportions of IgG+ MBCs (less than 0.5% of starting PBMCs). Thus, special care should be taken when comparing absolute IgG+ MBC numbers between subjects particularly with low starting frequencies of IgG+ MBCs, such as young children[12,14]. In such a case, expressing antigen-specific MBCs as a proportion of post-culture PBMCs gives even less insight into their actual *ex vivo* frequencies than when comparing across samples with a wide range of starting IgG+ MBC proportions. Expressing antigen-specific IgG+ MBCs as a proportion of IgG+ ASCs is therefore necessary to correct for this great variation in expansion. Prior knowledge of the starting concentration of IgG+ MBCs (e.g. by whole blood staining in a small volume prior to cryopreservation) might provide valuable additional information to accurately estimate differences in antigen-specific IgG+ MBC precursor frequencies: This information may be used to determine whether an actual difference in IgG+ MBC frequencies or MBC subsets exists between two groups, or whether they are comparable and differences in antigen-specific cells per total ASCs thus also reflect a different magnitude of the antigen-specific MBC response.

Another basic assumption of the MBC ELISpot assay is that all antigen-specific cell subsets have an equal ability to differentiate into ASCs. In our hands the

number of post-culture surface IgG+ B cells, however, did not fully predict the number of IgG+ ASCs. One potential reason could be the down-regulation of surface IgG expression on differentiating ASCs in mitogen-culture. This would lead to an underestimation of IgG+ ASCs, which could be rectified by performing intracellular IgG staining post culture. However, after mitogen-culture the number of surface IgG+ B cells slightly but significantly exceeds, and thus over- rather under-estimates the number of IgG+ ASCs. While we can further not formally show that all expanded IgG+ B cells are indeed plasma blasts (since staining for CD27 was not performed in addition to CD38), we find that the number of post-culture IgG+ cells and IgG+ ASCs correlates (Spearman r= 0.48, p<0.0001), indicating that at least a major proportion of those cell populations does overlap. Moreover, these data indicate that not all IgG+ B cells (*ex vivo* and post culture) actually acquire ASC function, as previously shown on single-cell level for isotype-switched MBCs after stimulation with CpG and cytokines[20,21]. A marker identifying MBC subsets with the capacity to become ASCs is unfortunately still lacking[20], and this may further differ depending on the combination of stimuli chosen: Since the description of the MBC ELISpot by Crotty *et al*.[5], many different polyclonal stimulation protocols relying on the activation of Toll-like receptors as well as cytokine receptor signaling have been tested to effectively differentiate MBCs into ASCs. Here we choose a stimulation cocktail containing PWM, SAC, CpG and IL-10, which was previously shown to be the most efficient out of 12 different stimulation protocols tested[6]. Based on the data we provide, future studies using other effective stimulation protocols (e.g. IL-2 and R848[9,11], or IL-15 and CpG[8]) or purified B cells[18] should determine whether the size and composition of the *ex vivo* B cell pool influences the expansion of total and IgG+ B cells during culture.

Most IgG+ B cells *ex vivo* are cMBCs, and their proportion correlated as strongly with IgG+ ASCs detected by ELISpot as the proportion of total *ex vivo* IgG+ B cells. Curiously, low proportions of IgG+ cMBCs within the B cell compartment *ex vivo* (and thus low numbers of IgG+ ASCs after mitogen-culture) were predominantly found in volunteers with high *ex vivo* ratios of atypMBC to cMBC. This was particularly evident for one volunteer, who at all three time points, at which PBMCs were sampled, showed the highest number of IgG+ B cells after mitogen-stimulation but only very low IgG+ ASC numbers, suggesting that only a small fraction of the efficiently expanded IgG+ B cells actually secreted IgG: this volunteer had a very low proportion of cMBCs and CD27- MBCs within IgG+ B cells, but a particularly large proportion of atypMBCs prior to mitogen-culture. This observation would be consistent with previous studies that reported atypMBCs

from HIV-infected individuals to be less efficient in differentiating into IgG+ ASCs *in vitro*, and similar data are available for a very limited number of atypMBCs from malaria-exposed subjects[10,22]. On the other hand, there is evidence that atypMBCs from malaria-exposed individuals may contribute to antibody production, since as for cMBCs, their BCR sequences can be matched to circulating plasma antibodies[23]. Additionally, bulk-sorted atypMBCs in this study contained both the membrane-bound and secreted form of IgG, however, a contamination of this population with non-atypMBC antibody secreting cells cannot be fully excluded, since CD38hi cells were not depleted[23]. It therefore remains to be conclusively established whether atypMBCs indeed actively secrete antibodies *in vivo*, or simply differentiate into ASCs like cMBCs (just potentially less efficiently) and then contribute to IgG-secretion upon antigenic stimulation. The extent to which atypMBCs and cMBCs in both malaria-exposed and healthy, malaria-naïve individuals vary in their ability to differentiate into ASCs to different stimuli also remains to be established. Should this indeed be the case, then data not only on the size, but also the detailed composition of the *ex vivo* IgG+ MBC pool might be helpful for data interpretation, especially when comparing populations with a broad range of proportions of atypMBC, such as individuals of different age groups in malaria-endemic areas or in different transmission settings[12,22].

Antigen-specific ASCs are often reported per million postculture PBMCs to get insights into the frequency of circulating antigen-specific MBCs. This readout, however, does not correct for differential expansion and thus skewing of the MBC compartment during mitogen-culture. Therefore only qualitative (higher or lower *ex vivo* frequencies of antigen-specific cells) but not quantitative differences between groups (which one aims to examine when using this readout) are preserved. This is specifically relevant for comparing antigen-specific MBCs between groups that markedly differ in size and composition of the B cell compartment, such as between different age groups[12,14]. A correction factor to accurately quantify antigen-specific MBC responses (taking into account both the size of the B cell compartment and the proportion of IgG+ MBCs *ex vivo*), would simplify analysis, but its determination is complicated by two main aspects: (i) different IgG+ MBC subsets may have varying abilities to differentiate into ASCs and (ii) other stimulation protocols may introduce different degrees of skewing during culture, for instance since receptor expression varies between MBC subsets[24]. In the absence of such a correction factor, we conclude that it is not possible to accurately estimate MBC frequencies by MBC ELISpot on an individual level. For this question, limiting dilution assay or *ex vivo* staining with fluorescently-labeled antigens[3,4] or B cell tetramers[2] remain the methods of choice.

An immediate practical implication of our finding is thus the way that results from bulk-culture MBC ELISpot should be reported: Specifically, we recommend not to express antigen-specific MBCs as a proportion of post-culture PBMCs in an attempt to compare their frequencies- particularly between clearly different cohorts, since this actually introduces bias. Instead, to allow interpretation of MBC ELISpot data in a meaningful manner, we propose to always include total IgG+ ASCs as a reference point to correct for variations in MBC precursor frequencies and hence expansion during mitogen-culture between donors. Between relatively homogenous groups (in regards to their B cell compartment), expressing antigen-specific cells per total ASCs is currently the best measure to correct for mitogen-culture effects. The same is true for longitudinal studies where the size and composition of the (memory) B cell compartment does not alter over time (as in the samples analyzed herein). In settings, however, where groups differ in the size and makeup of their (IgG+ memory) B cell compartment (or these parameters vary over time in longitudinal studies) due to age or pathogen exposure, we propose that this information about the B cell compartment should be additionally provided as a separate measure. This will also inform the reader appropriately whether it is simply the size of the MBC compartment that differs between (age) groups or alters over time, or the proportion of antigen-specific cells within the MBC compartment, or both- information that is lost when only providing the mitogen-culture biased readout of antigen-specific MBCs per post-culture PBMCs.

Conclusions

The number of total IgG+ ASCs detected by ELISpot after mitogen-stimulation correlates with the proportion of IgG+ MBCs *ex vivo*, highlighting the general robustness of this assay to compare MBC responses between different cohorts, i.e. at group level. The expansion of total and IgG+ B cells during mitogen-stimulation, however, was not identical in all cultures, but influenced by both the size and composition of the *ex vivo* B cell compartment, which vary widely between individuals. The uncorrected readout of antigen-specific MBCs per million post-culture PBMCs therefore only preserves the quality, but not the quantity of differences in the *ex vivo* MBC response between samples and thus groups or time points. Expressing antigen-specific cells per IgG+ ASCs is currently the best measure to correct for differences in the *ex vivo* B cell compartment and resulting mitogen-culture effects. To be able to evaluate not only changes within the MBC compartment, but their actual magnitude within the circulation, additional information on the size and composition of the *ex vivo* B cell compartment should be supplied as a separate measure, particularly under circumstances when the proportions IgG+ MBC are highly variable or proportions of B cell subsets are altered by environmental factors either between groups or over time.

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Supporting Information

Table S1 | B cells, IgG+ B cells and MBC subsets in Study A *ex vivo* samples over time

Subset	Group	Baseline	C-1	C+35
Total B cells[a]	Immunized[c]	**6.0** [3.7-6.6]	**5.4** [3.5-8.9]	**4.5** [3.8-7.4]
	Controls[d]	**4.8** [3.8-6.0]	**4.7** [4.4-5.7]	**5.0** [3.6-5.5]
IgG+ B cells[b]	Immunized	**6.6** [4.4-11.6]	**5.5** [3.9-10.5]	**6.5** [4.5-11.8]
	Controls	**7.5** [4.7-9.7]	**7.1** [4.4-10.3]	**6.4** [3.4-11.0]
cMBC[b]	Immunized	**13.5** [11.2-19.5]	**12.9** [11.2-16.1]	**12.4** [11.0-18.2]
	Controls	**17.3** [7.7-20.6]	**19.5** [7.7-22.8]	**14.7** [6.1-19.4]
CD27- MBC[b]	Immunized	**7.7** [5.9-11.4]	**7.2** [5.7-11.3]	**8.1** [6.0-11.0]
	Controls	**6.3** [4.6-7.5]	**5.8** [4.9-7.9]	**5.3** [4.1-7.7]
actMBC[b]	Immunized	**1.5** [0.8-2.0]	**1.0** [0.7-1.5]	**1.7** [1.1-2.1]
	Controls	**1.7** [0.7-1.3]	**1.2** [1.1-1.8]	**1.8** [0.8-3.5]
atypMBC[b]	Immunized	**2.4** [1.7-3.1]	**1.9** [1.1-4.1]	**2.3** [1.5-2.9]
	Controls	**2.3** [1.5-3.7]	**2.1** [1.4-2.8]	**2.3** [1.6-3.5]
nsMBC[b]	Immunized	**8.0** [4.5-11.2]	**8.5** [4.6-11.0]	**8.2** [5.3-11.3]
	Controls	**9.6** [8.8-12.0]	**9.0** [7.1-14.0]	**8.8** [7.8-13.0]

[a] Analyzed as percentage of viable PBMCs; median with interquartile range
[b] Analyzed as percentage of total B cells; median with interquartile range
[c] n=14
[d] n=10

Table S2 | B cells, IgG+ B cells and MBC subsets in Study B, *ex vivo* samples over time

Subset	Group	Baseline	I(1)+28	I(2)+28	I(3)+28	C-1	C+35	C+140
Total B cells[a]	Immunized[c]	**7.3** [4.2-8.5]	**5.4** [3.9-7.0]	**6.0** [3.7-6.9]	**5.2** [4.1-7.4]	**6.9** [4.6-9.2]	**7.8** [5.4-10.0]	**7.7** [5.0-9.1]
	Controls[d]	**7.1** [5.4-9.2]	n.d.	n.d.	n.d.	**7.8** [5.0-8.6]	**7.6** [6.6-9.4]	**6.9** [5.7-9.5]
IgG+ B cells[b]	Immunized	**8.0** [6.3-12.7]	**8.1** [6.0-12.1]	**7.9** [6.4-10.0]	**8.4** [6.4-13.0]	**8.2** [5.6-11.5]	**8.1** [6.0-11.9]	**8.3** [5.6-11.5]
	Controls	**7.5** [5.2-11.3]	n.d.	n.d.	n.d.	**6.3** [5.0-12.1]	**7.0** [4.6-10.3]	**6.7** [5.7-11.4]
cMBC[b]	Immunized	**13.8** [10.1-18.6]	**13.2** [9.4-18.1]	**13.6** [9.7-16.9]	**13.1** [10.0-19.0]	**13.9** [11.1-18.4]	**12.7** [9.9-16.6]	**13.1** [10.2-17.4]
	Controls	**14.9** [12.2-19.7]	n.d.	n.d.	n.d.	**16.2** [11.1-20.0]	**14.2** [11.0-18.3]	**14.5** [11.9-19.0]
CD27- MBC[b]	Immunized	**6.0** [5.0-8.1]	**5.7** [4.5-8.0]	**5.6** [4.6-7.5]	**5.5** [4.7-7.9]	**5.6** [4.7-8.8]	**6.6** [5.1-8.6]	**5.5** [4.6-9.2]
	Controls	**6.6** [4.6-7.3]	n.d.	n.d.	n.d.	**6.7** [4.7-7.6]	**7.3** [4.6-8.3]	**7.0** [4.5-8.0]
actMBC[b]	Immunized	**1.9** [1.5-2.5]	**2.2** [1.4-3.2]	**2.3** [1.6-3.6]	**1.9** [1.4-2.7]	**1.6** [1.3-2.7]	**2.0** [1.4-2.4]	**1.5** [1.3-2.1]
	Controls	**2.2** [1.3-4.6]	n.d.	n.d.	n.d.	**2.3** [2.0-2.8]	**2.8** [2.2-2.9]	**2.3** [1.7-2.7]
atypMBC[b]	Immunized	**2.4** [1.7-3.2]	**2.3** [1.5-3.0]	**2.0** [1.4-3.1]	**1.9** [1.5-3.1]	**1.8** [1.0-2.8]	**1.9** [1.5-3.5]	**1.6** [1.4-3.4]
	Controls	**1.5** [1.3-2.1]	n.d.	n.d.	n.d.	**1.6** [1.0-2.5]	**2.0** [1.3-3.2]	**2.1** [1.5-2.3]
nsMBC[b]	Immunized	**8.6** [5.6-13.0]	**8.3** [5.5-13.2]	**7.6** [4.8-12.5]	**7.5** [5.2-11.1]	**8.0** [5.2-13.1]	**7.7** [5.7-12.0]	**6.9** [4.9-15.1]
	Controls	**11.7** [9.0-12.7]	n.d.	n.d.	n.d.	**11.1** [6.6-16.7]	**9.6** [6.9-14.5]	**12.8** [7.7-13.6]

[a] Analyzed as percentage of viable PBMCs; median with interquartile range
[b] Analyzed as percentage of total B cells; median with interquartile range
[c] n=14
[d] n=10
n.d. not done

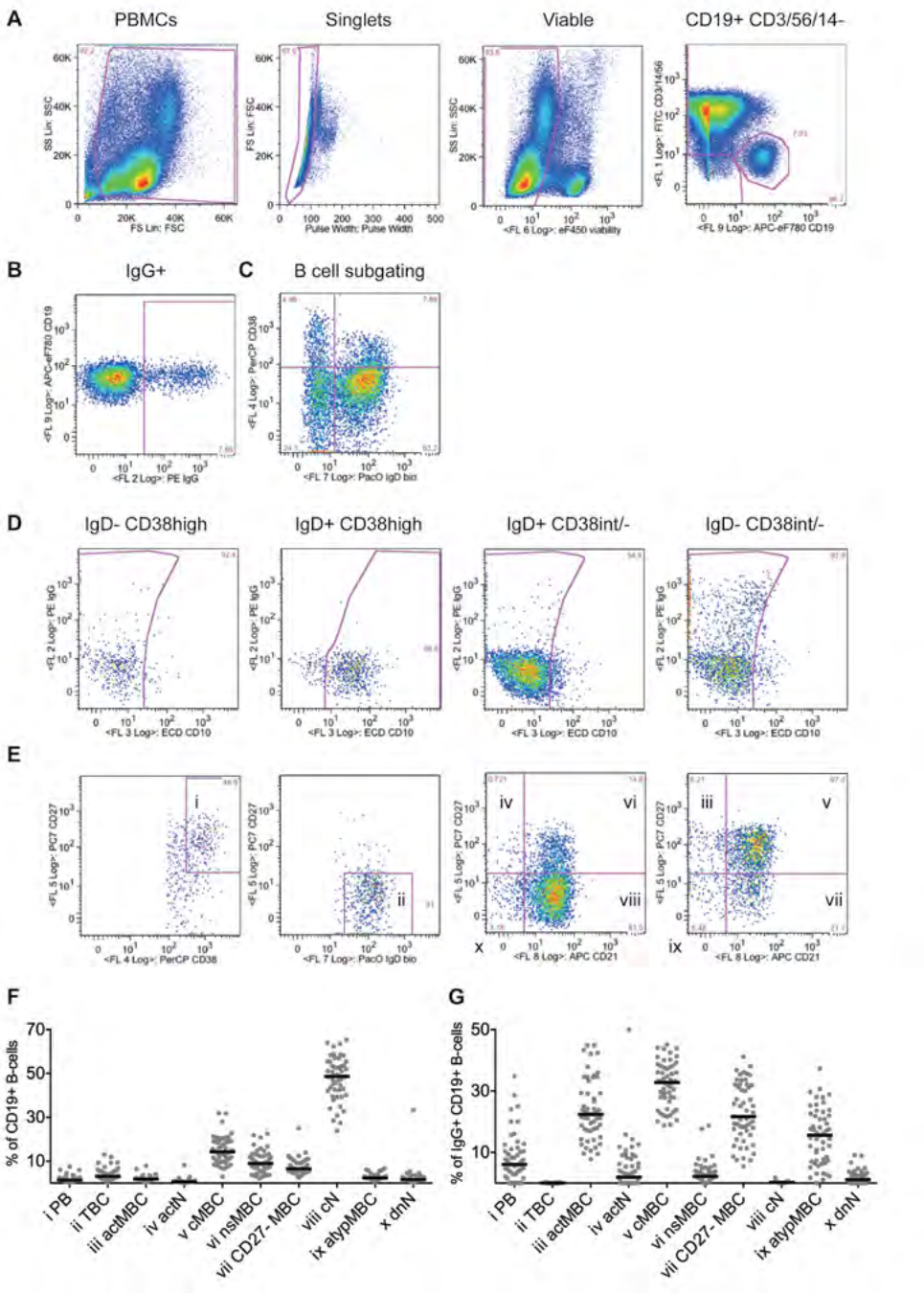

Figure S1 | Gating strategy of total and IgG+ B cells *ex vivo* *(for legend see next page)*

Figure S1 | Gating strategy of total and IgG+ B cells *ex vivo* *(see previous page)*
A) CD19+ B cell were identified following exclusion of debris, doublets, dead cells and CD3/CD56/CD14- cells. **B)** shows gating for IgG+ B cells. This IgG+ gate was later applied to individual B cell subsets, which were gated as follows: **C)** CD19+ B cells were first subdivided based on IgD and CD38 expression. **D)** CD10+ cells were excluded from all B cell populations except IgD+CD38hi B cells, which were specifically subgated based on CD10 expression. **E)** CD38hi B cells were divided into CD10-IgD-CD38highCD27+ plasma blasts (PB, i) and CD10+ IgD+CD38highCD272 transitional B cells (TBC, ii). CD38lowCD10- B-cells were subdivided into four pairs of switched/memory (IgD-) and non-switched/naive (IgD+) B cell populations: CD21-CD27+ activated MBCs (actMBC, iii) and activated naive B cells (actN, iv); CD21+CD27+ classical MBCs (cMBC, v) and non-switched MBCs (nsMBC, vi); CD21+CD27- MBC (CD27- MBC, vii) and classical naive B cells (cN, viii); and CD21-CD27- atypical MBCs (atypMBC, ix) and double negative naive B cells (dnN, x). **F)** shows the proportions of the ten B cell subsets of the total *ex vivo* B cell pool and **G)** the proportion of IgG+ cells within each individual B cell subset for the baseline samples of all 62 volunteers.

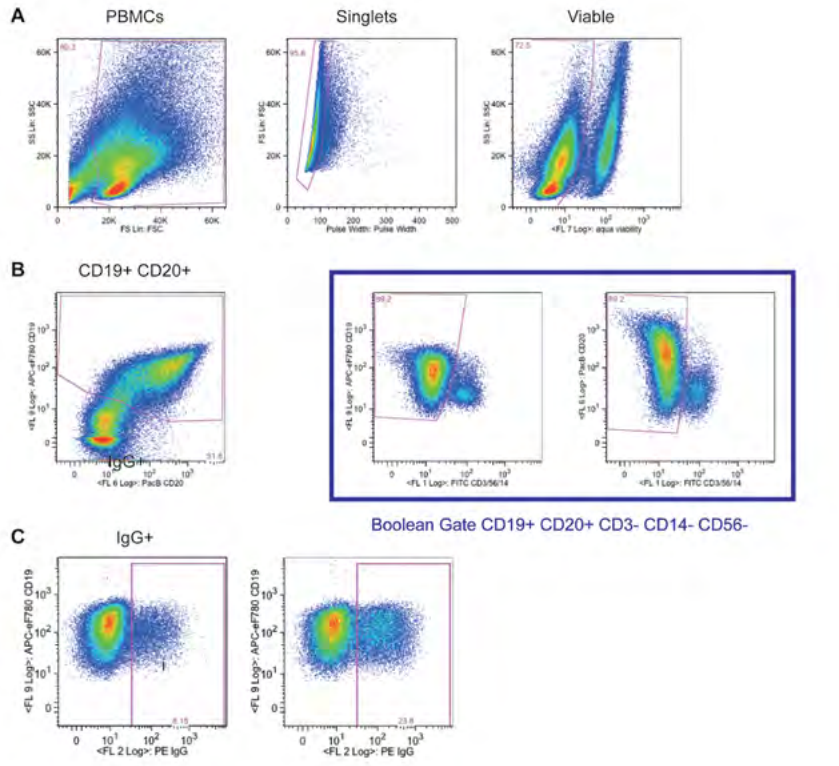

Figure S2 | Gating strategy of total and IgG+ B cells after 5 day mitogen-culture.
Following **A)** exclusion of debris, doublets and dead cells, B cells were identified by **B)** firstly gating on CD19+CD20+ cells and subsequently gating out CD3/CD56/CD14-cells (Boolean gating). **C)** shows gating for IgG+ B cells for two donors.

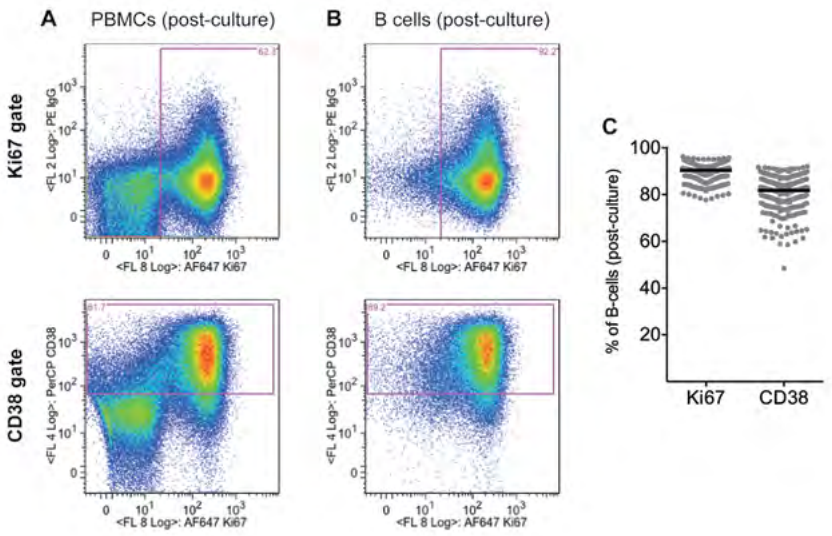

Figure S3 | CD38 and Ki67 expression on B cells after 5-day mitogen-culture
Ki67 and CD38 gates were based on negative populations (after exclusion of debris, doublets and dead cells) in **A)** total post-culture PBMCs and **B)** post-culture B cells. **C)** Grey dots show the percentage of Ki67 and CD38 positive cells within post-culture B cells cultures in all 269 stimulated samples, black lines indicate the median.

CHAPTER 6

Discussion and conclusion

Discussion

Despite elimination of malaria from temperate areas of Europe and North America, global eradication efforts in the 1950s have failed[1]. In Sub-Saharan Africa, South America and parts of Asia malaria is still a major public health concern[2]. In addition to the use of adequate vector-control as well as rapid diagnosis and treatment many believe that global eradication will only be possible once an effective malaria vaccine[3] will become available. A number of subunit and whole parasite vaccine strategies are pursued, but their success is hampered by the fact that it is still unclear what precisely constitutes protective antimalarial immunity[4]. It is therefore important to study immune responses in human and animal models. Chemoprophylaxis and sporozoite (CPS) immunization allows the vertebrate host to be exposed to sporozoites, infected hepatocytes and blood-stage parasites, while preventing pathogenic blood-stage densities with the antimalarial drug chloroquine. Three immunizations with 12-15 *P. falciparum* infected mosquitoes under chloroquine cover can induce sterile protection in human volunteers against a standard mosquito challenge with 5 infectious bites[5-7]. It was suggested that protection is mediated by pre-erythrocytic immunity. Evidence for human volunteers remains indirect since only peripheral blood-samples and *in vitro* assays can be utilized for validation. As pointed out by Plotkin[8] correlates of protection are often difficult to determine, especially if multiple correlates act in synergy, and are not absolute, meaning that even responses above a nominally protective level can lead to breakthrough infections in some individuals. Furthermore vaccine-induced responses are not necessarily the same that operate to clear the infection. For instance measles vaccination is aimed at inducing high antibody titers that were shown to correlate with protection in humans[9], but non-human primate models established that upon primary infection viremia in organs is largely controlled by CD8 T cells, the lack of which leads to measles infection even if immune serum from vaccinated animals was transferred[10,11]. Carefully designed animal models can thus be extremely helpful to fill the knowledge gap by addressing fundamental questions about the mechanism and not just the correlate of protection. Before protective mechanisms can be unraveled key components of immunization and the animal models used have to be considered, and where possible matched as far as possible to the human system.

The novel *P. chabaudi* mouse model of CPS immunization

We have developed a novel mouse model of CPS immunization using *P. chabaudi* as described in **chapter 3**. *P. chabaudi* shares many characteristics with *P. falciparum*[12], especially with regard to protection after re-infection. Since timing, route of infection[62,99] and antigen dose are critical for the initial priming of the antimalarial immune response[13-17], we employed similar immunizing conditions using infected mosquito bites, according to a recently published protocol describing the optimal conditions for *P. chabaudi* mosquito transmission by *A. stephensi*[18], as those in human CPS trials. This means that the exact dose of sporozoites and subsequently the number of infected hepatocytes will remain unknown. Interestingly salivary gland sporozoite numbers were dependent on the infecting *P. chabaudi* line where the AS line harboured a median of 438 (range 43-956) sporozoites, while the CB line produced 4 times more sporozoites (median of 1638, range 175-2576) per mosquito[18]. These sporozoite numbers are in the same order of magnitude as *P. falciparum* infection rates observed in wild mosquitoes[19-21], but 50 to 200 times lower than infection rates of experimentally infected *A. stephensi* for human CPS[6].

However, injection of sporozoites via mosquito bites is a stochastic process and does not necessarily correlate with the salivary gland load of the mosquito[22,23]. Typically few sporozoites (estimated between 1 and 100) are injected during one infectious bite[23]. Based on the number of infected erythrocytes observed directly after release from the liver and assuming that 10,000 merozoites are released from one infected liver cell, we estimate that approximately 5 hepatocytes (95% confidence interval (CI) 1-31) are infected with *P. chabaudi* AS, whereas in human CPS approximately 400 hepatocytes are *P. falciparum* infected after the first immunization (95% CI 137-1250)[6]. This may be a function of a higher number of infectious mosquito bites (usually 15[5-7]) in humans compared to a typical *P. chabaudi* mosquito transmission (Median 9.15 bites[18]). Therefore, we estimate that the number of infected hepatocytes after CPS immunization with mosquito bites is approximately 100-fold lower in the *P. chabaudi* mouse model than in human CPS.

It thus appears that the lower number of sporozoites in the salivary glands of *P. chabaudi* versus *P. falciparum* infected *A. stephensi* mosquitoes results in a lower number of infected hepatocytes in our mouse model compared with those in human CPS. This is likely due to a lower number of sporozoites being injected during mosquito bite, but low infectivity of *P. chabaudi* sporozoites in C57BL/6 mice could also contribute. Differences in infectivity were reported previously

between *P. berghei* and *P. yoelii*, the latter of which is by a factor of 2000 more infective to BALB/c mice than *P. berghei*, which was attributed to more effective innate immune responses that eliminate pre-erythrocytic *P. berghei* parasites[24]. To control the number of sporozoites establishing a liver-stage infection unequivocally, sporozoites have to be injected *iv*. This has been done in all other CPS immunization mouse models[25-33] but has so far not been attempted on a large scale for controlled human malaria infections[34]. If the number of sporozoites inoculated *iv* would be small (100 or less), whilst still reliably infecting people, this approach would model infection dynamics experienced by people in endemic areas giving rise to few infected hepatocytes. The smaller number of merozoites released would also make it possible to evaluate blood-stage vaccine candidates in the context of the whole life-cycle. *Iv* injection however has the disadvantage of bypassing the skin-stage of *Plasmodium*, where neutralizing antibodies could inhibit migration into the circulatory system[35,36] and priming of T cell responses may occur[37].

Another profound difference between human and rodent malaria parasites is the duration of liver-stage development. While *P. falciparum* typically egresses from hepatocytes 6.8 days after mosquito bite[38], *P. chabaudi* establishes a blood-stage infection after only 52h. Longer exposure to liver-stage parasites, as shown by immunization with irradiated sporozoites, is suggested to induce a broader repertoire of pre-erythrocytic immune responses[39], but this remains disputed[40]. It should be considered that the longer liver stage development in human *P. falciparum* (approximately 6.5 days) compared with those of rodent malarias (approximately 2 days) might influence the nature and magnitude of pre-erythrocytic immunity.

Large numbers of infected hepatocytes during CPS immunization induce pre-erythrocytic immunity

It is important to carefully consider the overall load of parasites in the liver, as our experiments clearly suggest that it is critical for acquisition of pre-erythrocytic immunity. After CPS immunization with *P. chabaudi* AS by infected mosquito bites, parasite burden in the liver was not different from unimmunized controls after mosquito-bite challenge. This does not appear to be because of the method of sporozoite administration as three *iv* injections of 100 sporozoites (the estimated equivalent number of sporozoites injected during *P. chabaudi* mosquito transmission[18]), also fail to induce pre-erythrocytic immunity. It therefore seems unlikely that sporozoites in the skin, which were suggested to induce immune

suppression, are responsible for the absence of protection after immunization with mosquito bites[16]. Our data also suggest that specific antibodies present in the skin[35] or CD8 T cells primed by sporozoites in draining lymph nodes[37] do not contribute significantly to pre-erythrocytic immunity after CPS immunization with a small number of sporozoites. However, if mice were CPS immunized three times with 100-times more sporozoites (10,000) injected *iv*, liver-parasite burden was reduced by over 90% following mosquito bite challenge. This is similar to reports from other rodent malaria CPS immunization studies using *P. berghei*[25-29] or *P. yoelii*[30-33], in which sterile pre-erythrocytic immunity is observed after immunization with high sporozoite doses (typically 10,000-50,000 sporozoites per immunization), while a reduction in sporozoite dose or the number of immunizations leads to breakthrough blood-stage infections upon challenge[30,32]. In human CPS immunization using bites of 5 mosquitoes on 3 occasions sterilely protected only 50% of volunteers[7] compared with almost 100% sterile protection after 3 immunizations with 15 infected mosquito bites[5-7]. After CPS with 5 bites only half of the volunteers had blood-stage parasitemia detectable by quantitative RealTime (qRT) PCR within the first cycle during the first CPS immunization, suggesting that fewer than 60,000 merozoites were released from the liver. This would imply that fewer than 6 hepatocytes were parasitized, which may explain the lack of protective immunity.

Another possibility is that protection depends on the ratio of immunizing and challenging mosquito bites: in the rodent model we use the same minimum number of bites (approximately 9) to ensure consistent infection of all mice for both immunization and challenge, whereas humans were immunized with 3 times more bites than required for 100% infection rate, which resulted in close to 100% sterile protection[5-7]. Equalizing the numbers i.e. using 5 bites for CPS immunization and challenge, reduced the rate of sterile protection to 50%[7]. It is not possible to increase the number of immunizing bites for mice because of animal welfare considerations; we therefore increased the number of immunizing sporozoites by *iv* injection. Keeping the number of mosquitoes constant in the challenge infection, we could show strong protection against liver-stage parasites can also be acquired with greater doses of sporozoites.

Taken together, evidence from the *P. chabaudi* mouse model described in **chapter 3** of this thesis, other rodent CPS models[25-33] and the human dose de-escalation trial[7], strongly suggests that the number of infected hepatocytes, which is determined by the number of sporozoites infecting the liver, is critical to determine whether pre-erythrocytic immunity is acquired.

Are large numbers of immunizing sporozoites required to elicit effective T cell-mediated pre-erythrocytic immunity?

A high parasite load in the liver may be necessary to induce large numbers of CD8 T cells[41], which have been shown to be the main mediators of pre-erythrocytic immunity[42]. Hepatocytes, in contrast to erythrocytes, express major histocompatibility complex (MHC) class 1 molecules, which allow interaction with cytotoxic T cells. One study suggests that antigen-specific CD8 T cells have to kill each infected hepatocyte individually by cognate interaction rather than by non-specific bystander effects mediated through inflammatory cytokines[43]. Previously however tumor necrosis factor (TNF), possibly acting via the induction of interleukin (IL) 6[45], and interferon (IFN) γ[44,45], was demonstrated to be crucial for the induction of pre-erythrocytic immunity, while the absence of Fas and Perforin, which can destroy cells upon directly contact, was reported to be dispensable for protection[46]. After immunization with irradiated sporozoites, at least 1% of all CD8 T cells have to be specific for the pre-erythrocytic parasite antigen circumsporozoite protein (CSP) to convey protection[47,48]. This is 100-1000 times greater than the frequencies of CD8 T cells necessary to protect against viral or bacterial agents[48], possibly because of the small proportion of infected hepatocytes present in the whole liver and the requirement to eliminate every single one of them to achieve sterile protection.

It seems that the burst size of the responding CD8 T cell population after the initial interaction of MHC I with parasite peptide is critical in determining the ultimate magnitude of the response[49,50]. Activation starts as little as 2h after initial contact with antigen-presenting cells[49,50]. Using transgenic mice with CD8 T cell receptors specific for *P. yoelii* CSP[51] it was shown that antigen-driven T cell activation occurs within the first 48 hours after initial exposure to infected hepatocytes[52]. Multiple subsequent immunizations do not seem to increase the frequency of transgenic CD8 T cells[52]. Immunization with one large dose of irradiated sporozoites apparently leads to higher CD8 T cell responses than life-long exposure in endemic areas[53,54]. Since natural exposure may result in a smaller number of sporozoites and infected hepatocytes experienced per infection[19-21], this may not lead to the acquisition of high frequencies of protective CD8 T cells, possibly explaining the apparent lack of pre-erythrocytic immunity in naturally-exposed populations[55].

Cytotoxic CD4 T cells, utilizing e.g. granzyme or perforin pathways, can also contribute to pre-erythrocytic antimalarial immunity in rodent models[56,57]. It was recently shown that the frequency of CD4 T cells expressing the degranulation marker CD107a are circulating at high frequencies in protected CPS immunized

volunteers[7]. As for CD8 T cells, the maximum response was already reached and maintained after one immunization[7], suggesting that the first exposure to parasite antigens also determines the magnitude of the CD4 T cell response.

Therefore both CD4 and CD8 T cells have been implicated in mediating pre-erythrocytic immunity after whole parasite immunization. Their induction seems to be crucially dependent on the initial immunization dose[53,54]. In the *P. chabaudi* CPS model, pre-erythrocytic immunity may hence be absent after immunization with low numbers of sporozoites, which are apparently insufficient to give rise to a sufficiently large effector memory T cell population.

Does cross-stage immunity contribute to protection after CPS immunization?

CPS immunization not only permits exposure to sporozoites and infected hepatocytes, but the use of chloroquine also allows parasites in erythrocytes to develop until the late trophozoite-stage[58]. The load of blood-stage parasites is hence another factor that will define generation of protective immunity[12,59]. Given orally, chloroquine treatment cleared blood-stage *P. chabaudi* parasitemia within 3 to 4 days in C57BL/6 mice, similar to humans[5-7]. The number of blood-stage parasites was quantified after each immunization by qRT PCR, which is to our knowledge the first time this has been determined in a mouse model. Chloroquine regimens used in other rodent CPS immunization models were retrospectively described to be sub-curative leading to blood-stage parasite persistence[30,31,33]. Doll *et al*. reported that parasite RNA was detectable in the spleen up to 60 days after immunization and chloroquine treatment in 50% of mice infected with *P. yoelii*[33]. In that case exposure to blood-stage parasites gave rise to T cell-independent blood-stage immunity[33]. Thus blood-stage parasite exposure (especially if prolonged) can lead to the induction of erythrocytic immunity. However given shared antigenic targets between blood- and liver-stage parasites[60] it also has the potential to alter the development of pre-erythrocytic protection following CPS immunization by inducing cross-stage immunity.

We showed that one self-cured infection initiated directly with *P. chabaudi* parasitized erythrocytes reduces liver parasite burden as efficiently (approximately 90%) as three CPS immunizations with 10,000 sporozoites. Together with evidence that immunization with attenuated blood-stage parasites can protect against sporozoite challenge[15,61,62] (although a reduction in liver-parasite burden was not always directly shown) it appears that exposure to blood-stage parasites may be a powerful way to induce pre-erythrocytic immunity. It may be that blood-

stage parasites can induce pre-erythrocytic immunity by boosting expansion of protective CD8 T cells as recently shown using a MHC I-restricted T cell receptor transgenic mouse line[63]. Also in humans T cell responses to blood-stage parasite antigens were always higher compared to sporozoites 2.5 years after CPS immunization, which could represent immunological cross-reactivity[64].
In our *P. chabaudi* CPS immunization model pre-erythrocytic immunity was not obtained after immunization with sporozoites by mosquito bite or with small numbers of sporozoites injected *iv*. Early abrogation of blood-stage parasitemia by chloroquine in CPS immunization[15] hence does not induce cross-stage protection, which was only achieved by prolonged blood-stage infection. However immunization with 10,000 sporozoites *iv* reduced liver parasite burden after challenge substantially. Since this reduction was less pronounced after immunization with irradiated sporozoites, which arrest early in liver-stage development, this raises the intriguing possibility that enhanced blood-stage parasite load after CPS immunization with 10,000 sporozoites compared to 100 sporozoites or mosquito bites, could contribute to liver-stage immunity. Similarly, induction of sterile protection in humans required only 30 to 45 mosquitoes using the CPS immunization regime, whereas 20 times more (over 1000) irradiated mosquitoes were needed[65,66]. Therefore blood-stage exposure during CPS immunization may contribute to the observed pre-erythrocytic protection.

The importance of cross-stage immunity for future malaria vaccine design

We consequently advocate in **chapter 2** that induction cross-stage protective immune responses should be considered, as this could enhance vaccine efficacy. Cross-stage immunity is likely facilitated by shared antigens between (late) liver and blood-stage parasites[60], which could be identified comparing protected and unprotected mice or volunteers using immunomics (whole *Plasmodium* genome or proteome-based antibody and T cell screening approaches)[67]. Immunization with *fabb*/*f*- genetically attenuated sporozoites that arrest late in liver-stage development can protect against direct blood-challenge demonstrating that cross-stage protective responses can also be elicited by pre-erythrocytic parasites targeting blood-stages[68]. Interventions that arrest parasite development early during the liver-stages, when the parasite proteome is less similar to that of the blood-stages[60], such as irradiation and some genetic modifications, have not been shown directly to protect against direct blood challenge. However, blood-stage parasites are recognized by T cells from volunteers immunized with irradiated

sporozoites[69].
Identification of the protective antigens that are shared between liver and blood-stage parasites deserve to be a focus for future malaria vaccine development. If protection is not stage-specific, a multi-stage malaria vaccine would not only reduce the number of pre-erythrocytic parasites substantially while abrogating disease caused by blood stage parasites, but exposure to one stage would also boost immunity against the other.

Blood-stage immunity following CPS immunization

We found that CPS induces partial blood-stage protection in our *P. chabaudi* model. Repeated exposure to blood-stage parasites during the immunization is the likely source of blood-stage immunity, although we cannot formally rule out that infected hepatocytes contributed by inducing cross-stage protective responses. Because of their small number after immunization with mosquito bites this seems however unlikely. Pre-patent period and parasitemia in the first 5 erythrocytic cycles are similar between immunized mice and controls, possibly because memory cells first have to be activated and an effective adaptive effector immune response has to be mounted. Blood-stage immunity was not strain-specific, since CPS immunization with *P. chabaudi* AS infected mosquito bites also protected against the more virulent CB strain.
Similarly, repeated immunizations with 100,000 *P. chabaudi* AS blood-stage parasites under atovaquone cover can elicit protection against blood-stage challenge with the more virulent *P. chabaudi* CB strain in mice[14] and protection was also observed after repeated immunization of human volunteers with approximately 30 erythrocytic parasites and atovaquone-proguanil treatment[70]. The latter findings may have however been confounded by persisting atovaquone levels[71]. Abrogation of blood-stage infections at different times by drug-treatment was also found to alter homologous and heterologous immunity to reinfection in mice[13,72], which was also demonstrated by analysing protection of neurosyphilis patients against repeated *P. falciparum* infection[73]. In endemic areas repeated exposure to malaria during infancy and childhood can also induce immunity against blood-stage parasites, which first reduces disease symptoms from severe to mild and can eventually control parasite densities, although sterile protection is rarely observed[4,74,75]. Together these data suggest that repeated exposure to blood-stage parasites generates protective immunity in a variety of experimental models and under field conditions.

In contrast to CPS-induced protection against the blood-stages of *P. chabaudi* shown in this thesis, no delay in patency or differences in blood-stage multiplication rates were observed in human CPS trials[6]. However, an effect on blood-stage parasitemia may have been missed because volunteers have to be treated with antimalarial drugs as soon as the first parasite is observed by thick blood film (typically around the 3rd erythrocytic replication cycle)[6]. One volunteer, classified as protected by the absence of a positive slide and no clinical symptoms, had 457 parasites per ml as determined by retrospective qRT PCR at 21 days post challenge just prior to presumptive drug treatment[6]. In addition to a profound reduction of liver-stage parasitemia and prolonged liver-stage development, erythrocytic immunity may have contributed to this delay. Conversely that this volunteer harboured over 1.3 million parasites in total (assuming 3l of blood) may also mean that erythrocytic immunity was not acquired.

Data from our new *P. chabaudi* mouse model reveal that CPS immunization can, in addition to pre-erythrocytic immunity induced after infection with high numbers of infected hepatocytes, also partially protect against blood-stage parasites, thus further enhancing the protective potential of this immunization regimen.

The importance of studying protection against blood-stage parasites in the context of mosquito transmission

After CPS immunization with *P. chabaudi* via infected mosquito bites, protection against both homologous and heterologous blood-challenge was only observed if blood-stage parasites were used from a donor-mouse infected by mosquito bite. Conversely CPS-induced blood-stage immunity was almost overridden when the parasites used for challenge had been serially blood passaged 26-32 times. For many years *Plasmodium chabaudi* parasites have been maintained in the laboratory by serial blood-passage i.e. injection of parasitized erythrocytes from one mouse into a naïve recipient. Thus the basic biology and immunology of blood-stage infections was studied in isolation from mosquito stages. This continuous, unnatural blood-passage, however, significantly increases parasite virulence for rodent[76,77] and non-human primate[78] malaria species. Spence *et al*.[79] demonstrated that a modification of the parasite itself took place by systematically comparing parasite virulence before and after mosquito transmission. Following mosquito transmission of *P. chabaudi,* blood-stage parasite growth is attenuated and signs of malaria pathology in mice (severe hypothermia, liver-damage, cachexia) are strongly reduced compared to serially blood passaged *P. chabaudi*

parasites[79]. Interestingly, despite low blood-stage parasite densities, mice infected by mosquito bite still suffered from severe anaemia and their infection was even more chronic (up to 90 days) than *P. chabaudi* infections after serial blood-passage[79]. Importantly attenuation after mosquito transmission was not due to low inoculation numbers or influenced by pre-erythrocytic parasite life-cycle stages, since the same results were observed after direct injection of mice with parasitized erythrocytes derived from a donor mouse infected by mosquito bite[79]. How different recently mosquito transmitted and serially blood-passaged parasitized erythrocytes are, was revealed by genome-wide RNA sequencing. Vector transmission changed 10% of the entire transcriptome, prominently up-regulating genes from large multi-gene families implicated in antigenic variation[79]. While only few members of a multigene-family (*Plasmodium* interspersed repeat genes (*pir*); termed *cir* in *P. chabaudi*) are transcribed after serial blood-passage, transcription of more than half of all *cir* genes was increased after mosquito transmission. This diversification of *cir* transcription is associated with a more protective immune response in the host in mosquito-transmitted blood-stage parasites, which in turn attenuates parasite virulence[79]. Whether this is a direct effect of *cir* expression is however not known[80]. Blood-stage parasites from mosquito-transmitted infections of *P. chabaudi* elicited innate and adaptive cellular responses earlier in infection, controlled systemic inflammation by reducing plasma levels of cytokines like IFNγ, TNF and IFNγ-inducible protein (IP) 10 and induced a stronger antibody response during the chronic phase of infection[79]. Given these profound differences in parasite transcriptome, elicited immune response and thus virulence, recently mosquito-transmitted blood-stage parasites should be used to evaluate protection in rodent protection models.

Evaluation of long-term protective efficacy

Another crucial factor for the evaluation of vaccine-induced protection is timing between immunizations and, perhaps more importantly, timing between immunization and challenge. Terminally differentiated effector cells are only transiently present and only a small proportion of the responding cells survive to become memory cells providing long-term protection[81,82]. In mice we chose 100 days after the last immunization, while this varied between 56[5]-133[7] days up to 2.5 years[64] in human CPS trials.

One hundred days after the last immunization mice immunized with *P. chabaudi* infected mosquito bites are partially protected against direct blood challenge and

mice immunized with large numbers of sporozoites injected *iv* have a strongly reduced liver parasite burden. In humans CPS immunized adults with 95% protection up to 4 months were re-challenged 2.5 years after initial immunization and 4 out of 6 were still sterilely protected against mosquito bite challenge[64]. Therefore CPS-induced immunity appears to be long lived. Any vaccine, but perhaps especially malaria, which has to be implemented in developing countries with major logistic challenges needs preferably to induce long-lived protection after a limited number of booster immunizations.

Blood-stage immunity after *P. chabaudi* CPS is largely B cell-independent

Antibodies are essential for the clearance of blood-stage parasites[83]. However in the acute phase of infection during the first month blood-stage parasitemia was indistinguishable between B cell-deficient mice (μMT)[84] and wild-type C57BL/6 mice. This was equally observed for a primary infection with recently mosquito transmitted *P. chabaudi* blood-stage parasites and after challenge infection of CPS immunized mice. Our findings are in line with data from *P. chabaudi* mouse models showing protection against re-infection in B cell-deficient mice using serially blood-passaged parasites[85,86]. It therefore appears that in the acute phase of infection cellular CD4 T cell responses control blood-stage parasitemia by secretion of cytokines (mainly IFNγ), macrophage activation and priming of the B cell response, which can then eliminate persisting parasites in the chronic phase[87-89].

We demonstrate that B cell-deficient μMT mice were not able to fully clear parasitemia during the chronic phase of infection and continuously maintained low-level parasitemia from 30 days post challenge until the end of the experiment 4 months later. Therefore antibodies are important in the chronic phase of infection to clear parasitemia[83]. Antibodies are also only induced in considerable quantities from day 30 after *P. chabaudi* infection[79]. Interestingly CPS immunized μMT mice had significantly lower blood-parasitemias even in the chronic phase compared to primary infected μMT mice. Therefore some antibody-independent immune mechanism must be triggered by CPS immunization. Since γδ T cells producing IFNγ are greatly expanded during chronic infection of μMT mice[83], the inflammatory environment they provide may be responsible for the observed reduction of parasitemia compared to primary infected μMT mice.

Pre-erythrocytic protection in human CPS is not associated with the magnitude of the antibody response against CSP, LSA-1, AMA-1 and MSP-1

Protective immunity induced by CPS immunization in humans is considered to be directed against infected liver cells[6]. Pre-erythrocytic immunity after immunization with irradiated and genetically attenuated sporozoites is largely CD8 T cell mediated[42]. Similarly T cell frequencies are higher in protected compared to unprotected CPS immunized volunteers suggesting that pre-erythrocytic immunity is dependent on cellular rather then humoral immune responses in this immunization regimen[7]. However it was recently shown[90] that antibodies may also have a functional relevance for pre-erythrocytic protection after CPS immunization of humans: purified IgG from CPS immunized volunteers can inhibit sporozoite traversal *in vitro* in a dose-dependent manner and reduces *P. falciparum* liver parasite burden in humanized mice[90]. It is however still unclear which property of the antibody response, i.e. inhibition of sporozoite invasion, antibody-mediated cellular killing or a different mechanism, is crucial. Furthermore not all antibodies specific for different parasite proteins will be equally protective. In **chapter 4** we therefore analysed in CPS immunized human volunteers the association of humoral responses to a set of immunodominant malaria antigens, all of which are considered to be potential vaccine candidates[91]. There was no association between protection and the magnitude of antibody responses against circumsporozoite protein (CSP), liver-stage antigen (LSA)-1, apical membrane antigen (AMA)-1 and merozoite surface protein (MSP)-1.

Monoclonal antibodies targeting the pre-erythrocytic antigen CSP were previously shown to confer complete protection against *P. berghei* sporozoite challenge in mice, the degree of which is dependent on the antibody concentration in serum and the number of sporozoites used for challenge[90]. There is of course the possibility that antibody responses against novel, less immunodominant antigens may be associated with protection[92]. Analysis of the plasma antibody profile from CPS[93] and irradiated[94] sporozoite-immunized volunteers shows a broad repertoire of antigens recognized. A combination of specific responses against multiple antigens rather than single antigens may afford protection. In natural transmission settings in Kenya a combination of high antibody titers against AMA-1, MSP-2 and MSP-3, but not MSP-1 or erythrocyte binding protein (EBA) 175, was predictive of protection from clinical malaria episodes[95]; high antibody responses against a combination of 5 out of the 10 top-ranked blood and cross-stage antigens, including novel antigens, indicated 100% protection from disease[96]. Conversely in a different study in a holoendemic area of Kenya high levels of IgG specific for

a combination of the pre-erythrocytic antigens CSP, LSA-1 and thrombospondin related anonymous protein (TRAP) reduced the risk of infection by 57%, whereas AMA-1, EBA175 and MSP-1 antibody levels did not correlate with protection[97]. In our study the combined antibody responses to the pre-erythrocytic and cross-stage antigens tested (CSP, LSA-1, AMA-1 and MSP-1) were not predictive of protection.

The question of which antibody specificities are responsible for the observed inhibition of sporozoites by IgG from CPS immunized volunteers[90] thus remains unanswered. Using new immunomics tools[67] both cellular and humoral responses should be thus analyzed to find correlates of protection, which can be then verified mechanistically *in vivo* in rodent models.

The magnitude of the antibody response is a sensitive predictor of exposure

While not predictive of protection, the magnitude of the humoral response against a set of 9 immunodominant antigens gave a sensitive indication of cumulative parasite exposure i.e. the number and dose of immunization and the life-cycle stage experienced. The magnitude of the antibody response increased stepwise with repeated exposure, which is in contrast to the reported development of the T cell response, where expansion seems to be completed in the first clonal burst[7]. Exposure to few *P. falciparum* infected erythrocytes for only one day[6] was accompanied by a strong boost of the MSP-1-specific antibodies. Thus boosting of MSP-1 antibody responses after challenge infection may be a sensitive marker of blood-stage exposure up to at least 140 days.

Short-lived plasma cells, which secret large amount of antibody in response to initial antigen encounter die within 3-5 days after an acute *P. chabaudi* infection as demonstrated by a drop in malaria-specific antibody titres[98]. Thereafter antibodies are secreted by long-lived plasma cells residing in the bone marrow and memory B cells, which differentiate into antibody-secreting cells upon antigen re-encounter, even in the presence of a chronic malaria infection[99]. The observed reduction of antibody titre against malarial antigens thus reflects the expected contraction of short-lived plasma cells in the absence of antigen i.e. parasite exposure. Memory B cells on the other hand are stably maintained over the course of several months between CPS immunization and challenge in the absence of parasite exposure. We can hence not find any indication that humoral immunity in this immunization regimen is impaired, as previously suggested in endemic settings[100,101] and some rodent malaria models as a result of high blood-stage parasitemias during

acute infection[102,103]. Repeated exposure to malaria therefore maintains both the antibody and memory B cell response, whereas the absence of exposure can lead to a reduction of the antibody response, but not the memory B cell response.

Conclusions

Both rodent and human CPS immunization models have contributed significantly to our understanding of antimalarial immunity. In this thesis we developed a novel *P. chabaudi* CPS immunization mouse model to determine the stage- and strain-specificity of protection. We establish unequivocally that pre-erythrocytic immunity after CPS immunization is dependent on a large number of sporozoites infecting hepatocytes. After immunization using the natural route of transmission by *P. chabaudi* infected mosquito bites, mice were protected against homologous and heterologous blood-challenge. Thus CPS immunization can induce pre-erythrocytic as well as strain-transcending blood-stage immunity, making it an even more powerful tool to study antimalarial immunity and for the identification of protective malaria antigens.

We also show that prolonged exposure to blood-stage parasites can reduce liver-parasite burden after mosquito-bite challenge. Future malaria vaccine development should consider the possibility that immunity to malaria is not stage-specific, but that responses against different stages can actually complement and enhance each other.

Using samples from CPS immunized human volunteers we could further demonstrate that antibody and memory B cell responses against few well-studied malaria antigens do not predict protection form challenge infection, but instead reflect exposure to the different life-cycle stages of the malaria parasite. That antibodies are dispensable for initial protection against blood-stage *Plasmodium* infection was futher verified using mice CPS immunized with *P. chabaudi*.

The novel *P. chabaudi* CPS immunization model presented in this thesis can be used in the future to dissect mechanisms and test correlates of protection. The complementary use of carefully characterised animal models and human CPS immunization is essential to address what constitutes protective antimalarial immunity and will thus crucially inform multi-stage malaria vaccine development.

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CHAPTER 7

Summary

Zusammenfassung

Samenvatting

Summary

Malaria continues to be a major global health concern despite interventions like vector control, rapid diagnosis and drug-treatment and many believe that elimination or eradication measures will only succeed with a highly effective malaria vaccine. However even after decades of research such a vaccine remains elusive. Vaccines based on individual malarial antigens are moderately effective at best and often convey no protective efficacy at all in endemic areas. Whole parasite vaccination approaches, which allow exposure to many parasite antigens, should hence be used to address precisely what constitutes protective antimalarial immunity; a question to which answers remain surprisingly vague. Immunity to malaria can be sterile i.e. directed against pre-erythrocytic life-cycle stages (sporozoites injected during mosquito bite or liver-stage parasites) or mimic naturally acquired immunity controlling blood-stage parasite densities and disease severity. Pre-erythrocytic vaccines suffer from the threat of breakthrough blood-stage infection causing (severe) disease, while blood-stage vaccines are designed to control blood-stage parasitemia and therefore disease severity, but allow on-going transmission to the mosquito vector. In **chapter 2** we therefore advocate a combination of these two approaches into a multi-stage malaria vaccine in order to be optimally safe and effective. Since many antigens are shared between liver and blood-stage parasites, immunity to malaria is not necessarily life-cycle stage-specific. We present evidence of cross-stage immunity from whole parasite pre-erythrocytic and blood-stage vaccination approaches. We show that functional protective cross-stage antigens exist and should be identified in future studies to promote multi-stage malaria vaccine development.

Chemoprophylaxis and sporozoite (CPS) immunization, which induces protection against malaria in humans with unprecedented efficiency, is one of the most powerful tools to investigate cross-stage immunity and to identify cross-stage antigens, as prophylactic chloroquine treatment allows exposure to both pre-erythrocytic and blood-stage parasites. Because obvious ethical constraints preclude the investigation of liver parasite burden in humans, **chapter 3** introduces a new mouse model of CPS immunization using *Plasmodium chabaudi* to investigate the stage- and strain-specificity of CPS-induced immunity. This model was designed to better mimic conditions of human infections. We found that immunization with *P. chabaudi* by mosquito bite under chloroquine cover protects against homologous and heterologous blood-stage parasite challenge. In contrast to human CPS studies, however, it does not generate pre-erythrocytic immunity. This may relate to the likely higher sporozoite numbers experienced by human volunteers after CPS immunization. Strikingly, pre-erythrocytic immunity can also be afforded by prolonged exposure to blood-stage parasites in the *P. chabaudi* mouse model. Therefore extending exposure to blood-stage parasites elicits cross-protective immune responses targeting pre-erythrocytic parasites, which can further enhance protective efficacy of CPS immunization. Our results highlight how complex the development of immunity against the different life-cycle stages of the malaria parasite is.

CPS immunized B cell-deficient mice showed the same level of protection in the acute phase of infection as mice with a normal immune system, clearly demonstrating that protective immunity to the early stages of the erythrocytic infection generated by this immunization is largely antibody-independent. These findings complement data in **chapter 4**, where we analyse humoral responses in peripheral blood samples of human volunteers enrolled in two clinical CPS immunization trials. Antibody and memory B cells against well-characterized, immunodominant pre-erythrocytic and cross-stage antigens are efficiently acquired over the course of CPS immunization. The magnitude of the humoral response is dictated by cumulative parasite exposure i.e. the number of immunizations and blood-stage parasite densities experienced during immunization. However, the magnitude of humoral responses to CSP, LSA-1, MSP-1 and AMA-1 does not predict protection from challenge infection. Functionality of these antibodies or responses to novel antigens may still account for a humoral component of CPS-induced protection, while it is equally possible that other immune responses like the induction of cytotoxic T cells exclusively mediate protective immunity.

To evaluate human humoral responses after CPS immunization we used the memory B cell ELISpot assay. Since this technique relies on a polyclonal pre-activation step that differentiates quiescent memory B cells into antibody-secreting cells, our concern was that antibody-secreting cells measured in ELISpot would not reflect memory B cell frequencies *ex vivo*. Therefore **chapter 5** provides a comprehensive flow cytometry-based analysis of B cell frequencies before and after mitogen stimulation. While the number of antibody-secreting cells detected in ELISpot is overall a good estimate of *ex vivo* memory B cell frequencies, size and composition of the *ex vivo* memory B cell compartment influences its expansion during culture. To correct for these differences antigen-specific memory B cell frequencies should therefore be expressed as percentage of antibody-secreting cells. In doing so the assay allows a good estimate of memory B cell frequencies *ex vivo*, but care should be taken when comparing between age groups or intervention strategies that may influence the composition of the memory B cell

compartment.
Taken together the combined findings using samples from both CPS immunized human volunteers and from a novel mouse model, show that i) CPS-induced immunity induced by relatively low dose of sporozoites is likely to be independent of antibody, ii) that pre-erythrocytic protection may rely on higher immunization dose sporozoites and iii) that protective cross-stage immunity can be induced. In **chapter 6** these findings were discussed in a broader context paying special attention to the influence of dose, route of administration and timing of immunization and challenge on the acquisition of antimalarial immunity after CPS immunization. This thesis therefore gives important insights into our understanding of immunity to malaria supporting clinical multi-stage malaria vaccine development.

Zusammenfassung

Malaria ist und bleibt eine globale Gesundheitskatastrophe, obwohl viele Anstrengungen, wie die Kontrolle von Mückenpopulationen, schnelle Diagnose und Behandlung mit effizienten Medikamenten, unternommen werden. Viele Wissenschaftler sind daher der Meinung, dass ein effektiver Impfstoff die einzige Chance ist Malaria zu besiegen. Doch obwohl seit Jahrzehnten daran geforscht wird, gibt es immer noch keine Impfung, die die Krankheit aufhält. Impfstoffe, die auf einzelnen Malaria-Antigenen basieren, induzieren bestenfalls moderate Immunität und viele versagen sogar komplett dabei, Menschen in endemischen Gebieten zu schützen. Da wir nicht wissen welche Antigene des Parasiten von zentraler Bedeutung sind um immun zu werden, ist es sinnvoll den ganzen Parasiten für Immunisierungen zu benutzen. Das erlaubt es dem Immunsystem in Kontakt zu allen Proteinen, die der Malaria Parasit exprimiert, zu gelangen. Erstaunlicherweise wissen wir immer noch nicht wirklich welche Immunmechanismen uns gegen Malaria schützen können.
Impfstoff-indizierte Immunität kann steril sein, also gegen die Leberstadien des Parasiten gerichtet, was klinische Krankheit verhindert. Alternativ kann sie den natürlichen Schutz gegen Parasiten in der Blutbahn imitieren, wie er von Erwachsenen in endemischen Gebieten erlangt wird. Beide Herangehensweisen haben ihre Nachteile: Impfungen gegen Leberstadien können, falls sie nicht 100% effektiv sind, einige Parasiten ins Blut gelangen lassen, die dort zu schwerer Erkrankung führen. Impfstoffe gegen Blutparasiten hingegen verhindern zwar Symptome, lassen aber gleichzeitig zu, dass infizierte Personen fortlaufend Malaria auf Mücken übertragen, welche dann erneut andere Menschen infizieren können. Eine Kombination beider Herangehensweisen, wie wir sie in **Kapitel 2** vorschlagen, wäre daher am sichersten und effektivsten. Da Leber- und Blutparasiten viele Antigene gemeinsam haben, muss Immunität nicht zwangsläufig spezifisch für ein bestimmtes Stadium sein. Und tatsächlich finden wir Hinweise auf stadienübergreifende Immunität nach Impfung mit ganzen Parasiten. Zukünftige Studien sollten deshalb diejenigen Antigene identifizieren, die für diese stadienübergreifende Immunität verantwortlich sind. Das Ziel dieser Herangehensweise ist es, einen Mehrstadien-Impfstoff zu entwickeln, der die Vorteile von Leber- und Blutstadien Impfungen vereint.
Chemoprophylaxe mit Sporozoiten (CPS) ist eine experimentelle, stadienübergreifende Immunisierungsstrategie, die Menschen mit bisher ungeahnter Effizienz gegen Malaria schützt. Da Immunität gegen Leberstadien nur indirekt in menschlichen Freiwilligen untersucht werden kann, stellen wir

in **Kapitel 3** ein neues *Plasmodium chabaudi* Mausmodell vor, mit dem wir die Stadienspezifität der Immunität nach CPS Immunisierung testen können. Dabei war es wichtig dieses Mausmodell so zu konzipieren, dass es klinische Studien mit Menschen in wichtigen Punkten repräsentiert. Wir konnten zeigen, dass Immunisierung mit *P. chabaudi* infizierten Mücken, bei gleichzeitiger prophylaktischer Behandlung mit dem Medikament Chloroquine, Mäuse vor einer Blutparasiteninfektion bewahrt, sie aber nicht gegen Leberparasiten schützt. Schutz gegen Leberparasiten war nur gewährleistet, wenn sehr große Mengen von Sporozoiten für die Immunisierung benutzt wurden. Darüberhinaus konnte eine längere Blutinfektion ebenfalls gegen Leberparasiten erfolgreich sein. Dies zeigt ganz klar, dass Immunität nicht stadienspezifisch ist. Eine Verlängerung der Dauer der Blutinfektion kann daher stadienübergreifende Immunität gegen Leberparasiten induzieren, was CPS Immunisierung noch erfolgreicher machen könnte.

CPS-immunisierte B-Zell-defiziente Mäuse zeigten das gleiche Schutzniveau in der akuten Phase der Malariainfektion wie Mäuse mit einem normalen Immunsystem. Das macht deutlich, dass Immunität im Blutstadium weitgehend Antikörper-unabhängig ist. Diese Ergebnisse ergänzen Daten in **Kapitel 4**, in dem wir humorale Reaktionen im peripheren Blut von Probanden von zwei klinischen CPS Studien analysierten. Antikörper und Gedächtnis-B-Zellen gegen Leber- und Mehrstadienantigene wurden effizient während der CPS Immunisierung erworben. Die Stärke der humoralen Antwort wird durch die Anzahl der Immunisierungen, die Immunisierungsdosis und die Anzahl der Blutparasiten während der Immunisierung, diktiert. Allerdings sagt die Stärke der humoralen Antwort gegen CSP, LSA-1, MSP-1 und AMA-1 nicht den Schutz vor einer erneuten Infektion voraus. Die Funktionalität dieser Antikörper oder Antikörper gegen andere Antigene könnten eine Rolle spielen, während es ebenso möglich, dass andere Mechanismen, wie z. B. zytotoxische T-Zellen, schützende Immunität vermitteln.

Um die menschliche B-Zell Antwort nach CPS Immunisierung zu bewerten, verwendeten wir den Gedächtnis-B-Zell ELISpot-Test. Da diese Technik auf einer polyklonalen Voraktivierungsstufe beruht, die ruhende Gedächtnis-B-Zellen in Antikörper-sezernierenden Zellen verwandelt, wollten wir sichergehen, dass die in ELISpot gemessenen Antikörper-sezernierenden Zellen Gedächtnis-B-Zellfrequenzen *ex vivo* widerspiegeln. **Kapitel 5** bietet daher eine umfassende Durchflusszytometrie-basierte Analyse der B-Zell-Frequenzen vor und nach Mitogenstimulation. Während Antikörper-sezernierenden Zellen im ELISpot-Test eine gute Schätzung der gesamten *ex vivo* Gedächtnis-B-Zell-Frequenzen

darstellen, beeinflusst die Größe und Zusammensetzung des *ex vivo* Gedächtnis-B-Zellkompartiment die Expansion während der Kultur. Um für diese Unterschiede zu korrigieren, sollten antigenspezifische Gedächtnis-B-Zellfrequenzen pro Gesamtanzahl der Antikörper-sezernierenden Zellen ausgedrückt werden. Dann erlaubt der ELISpot-Test eine gute Schätzung der Gedächtnis-B-Zell-Frequenzen *ex vivo*. Vorsicht ist hingegen geboten beim Vergleich zwischen den Altersgruppen oder Interventionsstrategien, die die Zusammensetzung des Gedächtnis-B-Zellkompartiments beeinflussen.

Die Erkenntnisse, die wir mit CPS-immunisierten Freiwilligen und einem neuen Mausmodell erworben haben, zeigen, dass CPS-induzierte Immunität gegen die frühen Stadien einer Blutinfektion wahrscheinlich Antikörperunabhängig ist, und dass der Schutz nicht spezifisch für ein bestimmtes Parasitenstadium ist. In **Kapitel 6** diskutieren wir diese Ergebnisse in einem breiteren Kontext mit besonderem Augenmerk auf den Einfluss der Dosis sowie Art und Zeitpunkt der Verabreichung für den Erwerb von Immunität nach CPS Immunisierung. Diese Doktorarbeit gibt daher wichtige Impulse für unser Verständnis wie Immunität gegen Malaria erworben wird, was für zukünftige Stadien-übergreifende Malaria Impfstoffentwicklung wichtig sein wird.

Samenvatting

Malaria blijft een wereldwijd gezondheidsprobleem ondanks interventies zoals vector controle, snelle diagnose en efficiente medicatie. Veel wetenschappers geloven daarom dat een effectief vaccin de enige manier is om malaria te bestrijden. Desalniettemin is er na tientallen jaren van onderzoek nog steeds geen werkend vaccin. Vaccins op basis van individuele malaria antigenen kunnen op hun best matige immuniteit induceren en zijn meestal niet werkzaam in endemische gebieden. Vaccinatie met complete parasieten, waarbij het immuunsysteem aan vele parasitaire antigenen wordt blootgesteld, moet dus worden gebruikt om een precies totaalbeeld van beschermende anti-malaria immuniteit te ontwikkelen. Verassend genoeg weet men nog steeds niet welk afweermechanisme ons zou kunnen beschermen tegen malaria. Immuniteit tegen malaria kan steriel zijn i.e. gericht tegen pre-erythrocytaire parasieten, of de natuurlijke bescherming tegen de bloedfase van de parasiet kan worden nagebootst, waardoor de mate van infectie beheerst kan worden. Beide benaderingen hebben nadelen: Als een vaccinatie tegen de leverfase niet 100% effectief is, kunnen parasieten in de bloedbaan terech komen en daar tot ernstige ziekte leiden, terwijl vaccinatie tegen de bloedfase de parasiet onderdrukt, maar nog steeds aan malariamuggen kan worden doorgegeven. Deze kunnen vervolgens opnieuw mensen infecteren. Een combinatie van beide benaderingen, een meerfasig malaria vaccin zoals beschreven in **hoofdstuk 2**, zou volgens ons de meest volledige, optimaal veilige en effectieve bescherming geven. Aangezien sommige antigenen worden gedeeld tussen de lever- en bloedparasieten, is immuniteit tegen malaria niet noodzakelijk fase-specifiek. Wij presenteren bewijs van fase overschrijdende immuniteit na complete parasiet pre-erythrocytaire en bloedfase vaccinaties. Toekomstige studies moeten antigenen identificeren die verantwoordelijk zijn voor deze fase overschrijdende immuniteit om een meerfasig malaria vaccin te ontwikkelen.

Chemoprofylaxe en sporozoiet (CPS) immunisatie is een experimentele fase overschrijdende immunisatie strategie, die bescherming induceert tegen malaria bij mensen met ongekende efficiëntie. Sinds leverfases uiteraard niet kunnen worden bestudeerd in menselijke vrijwilligers, presenteren we in **hoofdstuk 3** een nieuw *Plasmodium chabaudi* muismodel waarmee we de specificiteit van de immuniteit door CPS immunisatie kunnen onderzoeken. Dit muismodel is uiterst belangrijk om de condities voor menselijke klinische proeven zo goed mogelijk te weerspiegelen. Immunisatie door muggenbeten van *P. chabaudi* geïnfecteerde muggen onder chloroquine behandeling, beschermt tegen homologe en heterologe bloedfase parasieten, maar niet tegen de pre-erytrocytaire immuniteit.

Bescherming tegen de leverfase wordt alleen gegarandeerd als zeer grote hoeveelheden sporozoïeten worden gebruikt tijdens de immunisatie. Op deze manier kan CPS immunisatie pre-erythrocytaire en heterologe bloedfase immuniteit opwekken. Bovendien kan een langere blootstelling aan een bloedinfectie ook succesvol zijn tegen de leverfase. Hieruit blijkt weer dat de immuniteit niet fase-specifiek is, maar verschillende fases elkaar positief beïnvloeden en aanvullen. Daarom kan een uitbreiding van het blootstellen aan bloedfase parasieten, fase overschrijdende immuniteit tegen lever parasieten opwekken die CPS immunisatie nog succesvoller kunnen maken.

CPS geïmmuniseerde B-cel-deficiënte muizen vertoonden hetzelfde beschermingsniveau in de acute fase van infectie als muizen met een normaal immuunsysteem. Dit toont duidelijk aan dat bloedfase immuniteit opgewekt en gehandhaafd wordt door een grotendeels antilichaam-onafhankelijk mechanisme. Deze bevindingen werden ondersteund door data in **hoofdstuk 4**, waar we de humorale respons in perifere bloedmonsters van menselijke vrijwilligers, die deelnamen aan twee klinische CPS immunisatie proeven, analyseren. Antilichamen en geheugen B-cellen tegen goed gekarakteriseerde, immunodominante pre-erytrocytaire en fase overschrijdende antigenen zijn efficiënt opgewekt in de loop van CPS immunisatie. De sterkte van de humorale respons wordt bepaald door de cumulatieve parasitaire blootstelling i.e. het aantal immunisaties, de immunisatie dosering en de hoeveelheid bloedfase parasieten die zijn ervaren tijdens de immunisatie. De sterkte van de humorale respons op CSP, LSA-1, MSP-1 en AMA-1 wordt echter niet gecorreleerd met de bescherming tegen herinfectie. Functionaliteit van deze antilichamen of reacties tegen nieuwe antigenen kunnen een rol spelen in de humorale component van CPS geïnduceerde bescherming, terwijl het ook mogelijk is dat andere immuunresponsen, zoals de inductie van cytotoxische T-cellen, uitsluitend beschermende immuniteit bemiddelen.

Om de menselijke humorale respons na CPS immunisatie te evalueren, hebben we gebruik gemaakt van de geheugen B-cel ELISpot assay. Deze techniek berust op een polyklonale pre-activeringsstap die rustende geheugen B-cellen differentieert tot antilichaam afscheidende cellen. Wij maakten ons daarom zorgen dat antilichaam afscheidende cellen, gemeten met de ELISpot, geen geheugen B celfrequenties *ex vivo* zouden weerspiegelen. **Hoofdstuk 5** bevat een uitgebreide flowcytometrische analyse van B-cel frequenties voor en na mitogeen stimulatie. Terwijl antilichaam uitscheidende cellen gedetecteerd in ELISpot assays een goede schatting van het totale *ex vivo* geheugen B-cell compartiment weergeven, kunnen de omvang en samenstelling van het *ex vivo*

geheugen B-cel compartiment van invloed zijn gedurende de celkweek. Om voor deze verschillen te corrigeren zijn antigeen-specifieke geheugen B-cel frequenties uitgedrukt per antilichaam uitscheidende cellen. De ELISpot assay maakt dus een goede schatting van de geheugen B-cel frequenties *ex vivo* in de geanalyseerde CPS immunisatie bij gezonde volwassenen, maar er moet rekening worden gehouden met de vergelijking tussen leeftijdsgroepen en interventiestrategieën die de samenstelling van het geheugen B-cel compartiment kunnen beïnvloeden. Met de resultaten van CPS geïmmuniseerde vrijwilligers en het nieuwe muismodel hebben we aangetoond dat CPS-geïnduceerde immuniteit van een vroege bloedfase infectie waarschijnlijk antilichaam onafhankelijk is en deze bescherming niet fase-specifiek is. In **hoofdstuk 6** werden deze bevindingen besproken in een bredere context met bijzondere aandacht voor de invloed van de dosis, de wijze van toediening en het tijdstip van immunisatie, terwijl het de overname van de immuniteit na CPS immunisatie discussieert. Dit proefschrift geeft dus belangrijke inzichten in ons begrip van immuniteit tegen malaria, die belangrijk kunnen zijn voor de toekomstige meerfasige malariavaccin ontwikkeling.

Appendix

Acknowledgements

Bibliography

Curriculum vitae

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Apparently this section is the first (and often only) part people read when they hold this thesis in their hands. This seems a bit of a shame, since I have devoted four years of hard work and long hours of writing to make it as pretty as it is. So please take a look at the figures at least! Though in a way it does make sense that the acknowledgements are so important, because none of this work would have been possible without the help of some great people:

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Curriculum vitae

Wiebke Nahrendorf was born in Rodewisch (Germany) in 1987. She went to the University in Würzburg (Germany) to study Biomedicine with the aim of researching the biological principles of human diseases. During her Bachelor's degree she worked in the lab of Bertram Gerber in the Department of Neurobiology and Genetics; starting off as a student-assistant and later completing her Bachelor's thesis there. In her Bachelor's project she investigated brain functions important for learning and memory. While studying towards her Master's degree in Biomedicine, she moved internationally for two research internships. Her internship at the Wellcome Trust Sanger Institute in Cambridge (UK) with Oliver Billker, where she studied the function of calcium-dependent kinases in *Plasmodium berghei* ookinete motility, was her first contact with the malaria parasite. Her second internship took her to the National University of Singapore (Singapore), where she explored photoreceptor degeneration in the zebrafish model of *Retinitis pigmentosa* with Christoph Winkler.

For her future research she decided to focus on parasites and their interaction with the immune system of their hosts. At the Institute for Molecular Infection Biology, Würzburg (Germany), Heidrun Moll entrusted her with examining the role of interleukin 10 in cutaneous leishmaniasis infection for her Master's thesis. The knowledge that she gained during this project, both with regard to eukaryotic parasites and immunology, was the basis for her PhD project with Jean Langhorne and Robert Sauerwein, which is presented in this thesis. The PhD fellowship she received from the EviMalaR European Union Network of Excellence in Malaria Research allowed her to work both at the MRC National Institute for Medical Research in London (UK) and at the Radboud University Medical Centre in Nijmegen (The Netherlands). The focus of her PhD was to investigate immunity and protection from malaria after chemoprophylaxis and sporozoite immunization in both a mouse malaria model and samples from human volunteers participating in clinical trials.

In January 2015, she moved to Edinburgh (UK) to join the lab of Philip Spence at the Institute for Immunology and Infection Research as a postdoctoral research associate. Building on her unique PhD experience, she is now investigating how innate immune responses shape malaria disease severity with the aim to further her career in parasite immunology.